IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 4261
TRINH *et al.*	Art Unit: 1652
Appl. No. 09/306,986	Examiner: Hudson, R.
Filing date: May 7, 1999	Atty. Docket: 0942.4570001/RWE/FRC
For: **A Method for Synthesizing a Nucleic Acid Molecule Using a Ribonuclease**	

Brief on Appeal Under 37 C.F.R. § 41.37

Mail Stop Appeal Brief - Patents

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

A Notice of Appeal from the final rejection of claims 8-12, 56, and 70-73 was filed on September 30, 2005. Appellants hereby file this Appeal Brief, together with the required brief filing fee.

It is not believed that extensions of time are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 19-0036.

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I. Real Party In Interest

The real party in interest in this appeal is Invitrogen Corporation.

II. Related Appeals and Interferences

No other prior or pending appeals, interferences or judicial proceedings are known to the Appellants, the Appellants' legal representative, or assignee which may be related to, or directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

III. Status of Claims

Claims 8-13, 56, and 70-75 are pending in the application.

Claims 1-7, 14-55, and 57-69 have been canceled.

Claims 8-12, 56, 70, 71 and 73 are rejected[1].

Claims 13, 72, 74, and 75 are objected to[2].

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[1] Although the Office Action Summary mailed on June 3, 2005, indicates that claims 8-12, 56 and 70-73 are rejected, Appellants note that on page 3 of the Office Action, the Examiner states that "[c]laims 8-12, 56, 70, 71 and 73" are rejected. No other rejections are outstanding in this application. Thus, it appears that the inclusion of claim 72 among the rejected claims set forth in the Office Action Summary was in error.

[2] Although the June 3, 2005 Office Action indicates that claims 13, 74 and 75 are objected to, there is no outstanding rejection of claim 72. Thus, it appears that claim 72 should have been indicated as being objected to.

IV. Status of Amendments

No amendments were filed subsequent to the final rejection.

V. Summary of Claimed Subject Matter

Claim 8 is the sole independent claim involved in this Appeal. The invention defined by claim 8 relates generally to methods of synthesizing polynucleotides in the presence of ribonucleases. (*See* specification at page 2, lines 24-26). More specifically, the currently claimed invention relates to methods of synthesizing a nucleic acid molecule from a preparation comprising RNA and double-stranded DNA. The claimed methods comprise mixing the preparation of RNA and double-stranded DNA with one or more DNA polymerases and one or more peptides or polypeptides having ribonuclease activity. The peptides or polypeptides having ribonuclease activity are capable of degrading single-stranded RNA. The claimed methods further comprise incubating the mixture under conditions sufficient to synthesize a nucleic acid molecule complementary to all or a portion of the double-stranded DNA and under which the peptides or polypeptides having ribonuclease activity degrade the single-stranded RNA.

Support for claim 8 can be found throughout the specification, for example, at page 3, lines 18-26 through page 4, lines 1-14; at page 11, lines 21-23; at page 12, lines 21-23; at page 13, lines 1-9 and 13-16; at page 15, lines 10-12; at page 15, lines 23 through page 16, lines 1-12; at page 18, line 5 through page 19, line 5; and in Example 1 at page 25, line 15 through page 27, line 13.

VI. Grounds of Rejection to be Reviewed on Appeal

There is only one ground of rejection to be reviewed on appeal:

Claims 8-12, 56, 70, 71, and 73 stand rejected under 35 U.S.C. 103(a), as being unpatentable over Major, *Biotechniques 12*:40-43 (1992) (Exhibit 1) and Maudru *et al., J. Virological Methods 66*:247-261 (1997) (Exhibit 2). Appellants have traversed this rejection.

VII. Argument

A. Legal Standard for Obviousness

In proceedings before the Patent and Trademark Office, the Examiner bears the burden of establishing a prima facie case of obviousness based upon the prior art. *See In re Piasecki*, 745 F.2d 1468, 1471-73, 223 USPQ 785, 788 (Fed. Cir. 1984). To meet this burden, the Examiner must satisfy three requirements. First, all of the claim limitations must be taught or suggest by the prior art. *See In re Royka*, 490 F.2d 981, 984-85, 180 USPQ 580, 583 (CCPA 1974); *see also In re Glaug*, 283 F.3d 1335, 1341-42, 62 USPQ2d 1151, 1154 (Fed. Cir. 2002) (finding a claim not obvious because the prior art did not teach "spaced zones of adhesive" as recited in the claim); *In re Rijckaert*, 9 F.3d 1531, 1533, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993) (finding a claim not obvious because the prior art did not teach all claim limitations). Second, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teaching. *See In re Rouffet*, 149 F.3d 1350, 1357, 47 USPQ2d 1453, 1457-58 (Fed. Cir. 1998). Third, there must be a reasonable expectation of success. *See*

In re Merck & Co., Inc., 800 F.2d 1091, 231 USPQ 375 (Fed. Cir.1986). The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, not in Applicants' disclosure. *See In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

Evidence of a suggestion, teaching, or motivation to combine prior art references may flow, *inter alia*, from the references themselves, the knowledge of one of ordinary skill in the art, or from the nature of the problem to be solved. *See In re Dembiczak*, 175 F.3d 994, 999, 50 USPQ2d 1614, 1617 (Fed. Cir. 1999). Although a reference need not expressly teach that the disclosure contained therein should be combined with another, *see Motorola, Inc. v. Interdigital Tech. Corp.*, 121 F.3d 1461, 1472, 43 USPQ2d 1418, 1489 (Fed. Cir. 1997), the showing of combinability, in whatever form, must nevertheless be "clear and particular." *Dembiczak*, 175 F.3d at 999, 50 USPQ2d at 1617. "Board conclusory statements regarding the teaching of multiple references, standing alone, are not evidence." *Id.*, 175 F.3d at 999, 50 USPQ2d at 1617, *see also In re Kotzab*, 217 F.3d 1365, 1371, 55 USPQ2d 1313, 1318 (Fed. Cir. 2000) ("particular findings must be made as to the reason the skilled artisan, with no knowledge of the claimed invention, would have selected these components for combination in the manner claimed.")

B. The Cited References

1. The Major Reference

The Major reference discloses a PCR-based assay for screening point mutations. The method of Major involves the use of oligonucleotide primers, the 3'-terminal nucleotide of which may or may not be complementary to a specific nucleotide in a

target sequence. *See* Major at page 40, Figure 1. The principle behind this assay is that PCR amplification should occur only when the 3'-terminal nucleotide of a primer base-pairs with the corresponding nucleotide on the template nucleic acid, and should not occur when the 3'-terminal nucleotide of a primer does not base-pair with the corresponding nucleotide on the template nucleic acid. *See* Major at page 42, left column.

Counter to this expectation, Major observed in his assay that "Even bacterial colony lysates showed clear negative results with all three 3'-terminal mismatches; however T:T mismatches gave some extra minor bands." *See* Major at page 42, bottom center column. Thus, Major attributed the "extra minor bands" to amplification products produced from oligonucleotides that have 3'-terminal nucleotide mismatches. Major also noted that other researchers had observed PCR amplification despite the presence of a 3'-terminal nucleotide mismatch. *See* Major at page 42, center column (citing Wu *et al.*, *Proc. Natl. Acad. Sci. USA 86*:2757-2760 (1989) and Kwok *et al.*, *Nucl. Acids Res. 18*:999-1005 (1990)).

2. The Maudru Reference

The Maudru reference discloses an assay for detecting the presence of retroviral reverse transcriptases in a sample. *See* Maudru, sentence bridging pages 247-248. The assay involves a reverse transcription step in which a sample suspected of containing a retroviral reverse transcriptase is combined with an RNA template. Any cDNA produced is then amplified by PCR using a DNA polymerase. *See* Maudru at page 248, left column. The principle behind this assay is that only samples that contain a retroviral reverse transcriptase should produce cDNA and consequently PCR-amplified DNA,

while samples that do not contain a retroviral reverse transcriptase should produce no cDNA and consequently no PCR-amplified DNA.

Counter to this expectation, Maudru observed that "background" PCR amplification can occur even when cDNA is not produced from the reverse transcription step; as in control reactions that contain no retroviral reverse transcriptase. *See* Madru at page 256, left column. Maudru observed that this "background" could be eliminated by treating the sample with a ribonuclease to degrade the RNA template before adding the DNA polymerase for the PCR amplification step. *See* Maudru at page 258, left column. Thus, Maudru attributes the background amplification products to an intrinsic RNA-dependent DNA polymerase (*i.e.,* reverse transcriptase) activity of the AmpliTaq DNA polymerase used in the assay. *See* Maudru at page 256, left column.

C. The Examiner's Position

The outstanding obviousness rejection is based on the Examiner's assertions that (1) a person of ordinary skill in the art would have attributed the "extra minor bands" mentioned in the Major reference to the presence of contaminating RNA in the reactions; and (2) that a person of ordinary skill in the art would have been motivated to include a ribonuclease digestion step in the Major assay based on Maudru's teaching that "the background signal in a similar assay was found to be due to an intrinsic RNA-dependent DNA polymerase activity of the *Taq* DNA polymerase." Specifically, the Examiner has stated that:

> While Major does not attribute background difficulties to contaminating RNA, one of skill in the art would realize that given the employment of the method of Major to bacterial lysates, there would be a substantial amount of

> background RNA in the preparation. This knowledge in combination with that taught by Maudru et al. stating that the background signal in a similar assay was found to be due to an intrinsic RNA-dependent DNA polymerase activity of the *Taq* DNA polymerase would lead one of skill in the art who was attempting to successfully use a PCR method to screen for small mutations to include a ribonuclease digestion step prior to PCR amplification as a means of making the assay more sensitive. In support of the above, applicants attention is drawn to Major, page 42, middle column, which states "the present results indicate that all three possible terminal T mismatches can be equally discriminated under standard PCR conditions, especially when using mini-prep DNA". Such a statement clearly supports that even Major recognized the taught method had different results or sensitivities depending on the template used (noting the reference to "especially"), although Major did not comment on the specific difference of the two different types of template preparations. One of skill in the art would understand that the difference was likely the presence of contaminating material, such as RNA.

See Office Action dated March 17, 2004 at pages 8-9 (emphasis in original).

D. The Appellants' Position

Claims 8-12, 56, 70, 71 and 73 are not obvious over the Major and Maudru references.

1. The Examiner's Argument That a Person of Ordinary Skill in the Art Would Have Attributed the "Extra Minor Bands" Mentioned in the Major Reference to the Presence of Contaminating RNA in the Reactions is Incorrect

As discussed above, Major himself did not attribute the "extra minor bands" that he observed in his assay to contaminating RNA. Rather, he attributed the "extra minor bands" to amplification products produced from oligonucleotides that have 3'-terminal nucleotide mismatches. *See* Major at page 42, bottom center column. Major's attribution is consistent with the observations of other researchers who he noted had

observed PCR amplification despite the presence of a 3'-terminal nucleotide mismatch. *See* Major at page 42, center column (citing Wu *et al.*, *Proc. Natl. Acad. Sci. USA 86*:2757-2760 (1989) and Kwok *et al.*, *Nucl. Acids Res. 18*:999-1005 (1990)).

Despite the fact that Major himself, in a manner consistent with the observations with others, attributed the "extra minor bands" to something other than contaminating RNA, the Examiner's position is: (1) that a person of ordinary skill in the art would have recognized that bacterial colony lysates contain a substantial amount of RNA, (2) that Major indicates that better discrimination of terminal T mismatches was obtained using mini-prep DNA than with bacterial colony lysates; and therefore that (3) a person of ordinary skill in the art would have attributed the difference in terminal T mismatch discrimination to the presence of RNA in bacterial colony lysates.

The Examiner's reasoning is flawed and cannot support a *prima facie* case of obviousness. First, persons of ordinary skill in the art would have appreciated at the time of the effective filing date of the present application that bacterial colony lysates contain many factors other than RNA (*e.g.*, proteins, salts, lipids, signaling molecules, etc.), and that, like RNA, such factors are absent from mini-prep DNA. The Examiner has failed to explain why a person of ordinary skill in the art, considering all the factors found in bacterial colony lysates, would have specifically regarded RNA as the one factor responsible the difference in 3'-terminal T mismatch discrimination described in Major.

The Examiner's implication that a person of ordinary skill in the art would have believed that errors in terminal nucleotide mismatch discrimination occur only in bacterial colony lysates (which contain RNA) but not in other DNA preparations (which

do not contain RNA) is incorrect. To the contrary, it was well known in the art at the time of the effective filing date of the present application that errors in terminal mismatch discrimination (*i.e.*, extension of oligonucleotides having mismatched 3'-terminal nucleotides) can and do occur in purified DNA preparations lacking RNA.

For example, Kwok *et al.*, *Nucl. Acids Res. 18*:999-1005 (1990) ("Kwok") (Exhibit 3), describe assay systems involving the use of plasmid DNA or PCR-generated products as templates for PCR extension reactions. *See* Kwok, page 1001, left column. Using these samples – *which lacked RNA* – template amplification was observed using several primers having mismatched 3'-terminal nucleotides. *See, e.g.*, Kwok at page 1001, right column ("the presence of a T at the 3' end of the primer provided efficient amplification irrespective of the corresponding nucleotide in the template.") Kwok attributed these errors in terminal mismatch discrimination to factors such as dNTP concentration and primer length. *See* Kwok at page 1004, right column. (Kwok was cited in Major as reference No. 6.) Clearly, RNA could not have been a factor for the terminal mismatch discrimination errors described in Kwok.

Because it was well known in the art at the time of the effective filing date of the present application that errors in terminal mismatch discrimination occurred in samples that did not contain any RNA, a person of ordinary skill in the art would have appreciated that factors other than RNA were responsible for errors in terminal mismatch discrimination. A discussion of other factors reported to influence 3'-terminal mismatch discrimination is found in Charlieu, "Chapter 12, Distinction Between Almost-Identical DNA Sequences by Polymerase Chain Reaction," in PCR Technology Current Innovations, pp. 101-106, Griffin and Griffin Eds., (1994) (Exhibit 4). Factors

mentioned in Charlieu include the nature of the DNA template, the nucleotide concentration, $MgCl_2$ concentration, *Taq* DNA polymerase concentration, and the presence of chemicals such as tetramethylammonium chloride (TMAC) or Perfect Match. *See* Charlieu at pages 105-106. Charlieu concludes that "[t]he stringency of PCR is defined by a combination of these factors." *See* Charlieu at page 106. Nowhere is it suggested that RNA can influence 3'-terminal mismatch discrimination. Thus, a person of ordinary skill in the art would not have regarded the difference in terminal mismatch discrimination alluded to in Major as being caused by RNA in bacterial colony lysates.

The Examiner has presented no evidence whatsoever to indicate that, at the time of the effective filing date of the present application, persons of ordinary skill in the art would have regarded RNA as a factor which might interfere with nucleic acid synthesis reactions. In fact, Applicants note that the only document of record that indicates that RNA can interfere with nucleic acid synthesis is *Applicants' own specification*. *See, e.g.*, specification at page 2, lines 20-23. Applicants' own specification, however, cannot be used to establish a *prima facie* case of obviousness. *See In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

The Examiner has not pointed to any evidence to support the contention that, despite the large number and variety of factors that are found in bacterial colony lysates and not in mini-prep DNA, a person of ordinary skill in the art would have believed that RNA was the specific factor which caused the difference in terminal T mismatch discrimination mentioned in Major. Since this contention is the basis of the obviousness

rejection and there is no evidence to support it, a *prima facie* case of obviousness has not been established.

2. The Examiner's Argument That a Person of Ordinary Skill in the Art Would Have Been Motivated to Include a Ribonuclease Digestion Step in the Major Assay Based on Maudru's Teaching that "The Background Signal in a Similar Assay Was Found to be Due to an Intrinsic RNA-Dependent DNA Polymerase Activity of the Taq DNA Polymerase" is Incorrect

The Major and Maudru references relate to very different, non-analogous assay systems, and persons of ordinary skill in the art would have had no motivation to associate or combine their teachings. Furthermore, Maudru attributes the presence of background amplification products in an assay designed to detect reverse transcriptases to an intrinsic RNA-dependent DNA polymerase activity of the DNA polymerase used in the assay. These background amplification products arise for a completely different reason than the "extra minor bands" mentioned in Major, which were caused by the extension of primers having a 3'-terminal mismatch. Because the background amplification products in Maudru arise for an entirely different reason than the "extra minor bands" observed in the Major assay, persons of ordinary skill in the art would not have been motivated to apply Maudru's strategy to reduce background amplification to address the shortcoming of the Major's assay. That is, persons of ordinary skill in the art would not have been motivated to use a strategy shown to be effective to eliminate background amplification caused by an inherent RNA-dependent DNA polymerase activity to address the presence of "extra minor bands" caused by the extension of primers having 3'-terminal mismatches observed in the Major's assays. Persons of ordinary skill in the art would not have attributed the "extra minor bands" caused by the extension of primers having 3'-terminal mismatches to an RNA-dependent DNA

polymerase activity of *Taq* polymerase, especially since (as discussed above) such extension was known to occur in systems that lack RNA. Thus, a person of ordinary skill in the art would not have been motivated to combine the ribonuclease treatment step of Maudru with the assay of Major.

E. Conclusion

In view of the forgoing discussion, Appellants respectfully submit that the subject matter defined by claims 8-12, 56, 70, 71 and 73 is patentable over the cited art and that the Examiner has not met the burden of establishing a *prima facie* case of obviousness. Accordingly, Appellants respectfully request that the Board reverses the Examiner's final rejection of these claims under 35 U.S.C. § 103 and remand this application for issue.

Respectfully submitted,

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VIII. Claims Appendix

8. A method for synthesizing a nucleic acid molecule from a preparation comprising RNA and double-stranded DNA, said method comprising:

a) mixing the preparation with one or more DNA polymerases, and one or more peptides or polypeptides having ribonuclease activity, wherein said peptides or polypeptides having ribonuclease activity are capable of degrading single-stranded RNA; and

b) incubating said mixture under conditions sufficient to synthesize a nucleic acid molecule complementary to all or a portion of said double-stranded DNA and under which said peptides or polypeptides having ribonuclease activity degrade said single-stranded RNA.

9. The method according to claim 8, wherein said peptide or polypeptide having ribonuclease activity is selected from the group consisting of: RNase A, RNase T1, RNase S, RNase B, RNase C, RNase T2 and enzymatically active fragments, variants, derivatives or mutants thereof.

10. The method according to claim 8, wherein said mixture further comprises one or more components selected from the group consisting of: a) at least one nucleotide; b) at least one suitable buffer for nucleic acid synthesis; and c) at least one primer.

11. The method according to claim 8, wherein said DNA polymerase is thermostable.

12. The method according to claim 11, wherein said thermostable DNA polymerase is selected from the group consisting of: Taq DNA polymerase, Tne DNA polymerase, Tma DNA polymerase, Tth DNA polymerase, Tli DNA polymerase, Pfu DNA polymerase, Pyrococcus species GB-D DNA polymerase, Pwo DNA polymerase, Bst DNA polymerase, Bca DNA polymerase, Tfl DNA polymerase and enzymatically active fragments, variants, derivatives or mutants thereof.

13. The method according to claim 10, wherein one or more of said nucleotides are detectably labeled.

56. The method of claim 8, wherein said preparation is from any cell or tissue selected from the group consisting of bacteria; insect; bird; fish; plant; yeast; prokaryote; eukaryote; and mammals.

70. A method according to claim 8, wherein said double-stranded DNA comprises an expression vector.

71. A method according to claim 8, wherein said double-stranded DNA comprises a cloning vector.

72. A method according to claim 8, wherein said double-stranded DNA comprises genomic DNA.

73. A method according to claim 8, wherein said double-stranded DNA comprises a plasmid or a cosmid.

74. A method according to claim 8, wherein said double-stranded DNA comprises viral DNA.

75. A method according to claim 8, wherein said double-stranded DNA comprises phage DNA.

IX. Evidence Appendix

Exhibit	Title of Exhibit	Location in Record
Exhibit 1	Major, *Biotechniques 12*:40-43 (1992)	Cited by Examiner in Office Action dated February 11, 2003
Exhibit 2	Maudru *et al., J. Virological Methods 66*:247-261 (1997)	Cited by Examiner in Office Action dated January 2, 2001
Exhibit 3	Kwok *et al., Nucl. Acids Res. 18*:999-1005 (1990)	Submitted by Applicants with Amendment and Reply Filed on June 17, 2004, entry of which was directed by way of Request for Continued Examination, filed on September 8, 2004
Exhibit 4	Charlieu, Ch. 12 in *PCR Technology Current Innovations*, pp. 101-106 (1994	Submitted by Applicants with Amendment and Reply Filed on June 17, 2004, entry of which was directed by way of Request for Continued Examination, filed on September 8, 2004

X. Related Proceedings Appendix

None.

A Rapid PCR Method of Screening for Small Mutations

ABSTRACT

We report a modified method of screening for point mutations using a PCR approach based upon the sensitivity of PCR to the 3′ terminus of the primer. This method provides a sensitive screen when using either plasmid DNA or bacterial cell lysates. We have optimized the technique for general use to allow rapid screening of mutants with good discrimination. Unlike previous similar methods, this technique has no inherent limitation in primer design on the 3′-terminal base chosen.

INTRODUCTION

The advent of PCR has facilitated rapid and novel methods for site-directed (3,4,9) and regional mutagenesis (2). One of the most time-consuming tasks in any mutagenesis procedure involves ascertaining the presence of the desired mutation(s) within the mutated fragment or within some vector into which the mutated fragment is subsequently cloned. Traditional screening employs methods such as diagnostic restriction enzyme digestion, oligonucleotide hybridization screening and direct sequencing of the target DNA. These methods are often time-consuming, dependent on diagnostic restriction enzyme sites or necessitate carefully controlled conditions to discriminate single nucleotides. More recently a number of PCR-based mutant detection methods have been proposed, including ligation amplification (7), single nucleotide primer extension (sNuPE) (5) and allele-specific PCR (ASPCR) (1,8,10, 11). ASPCR has been successfully used, for example, in the direct detection of the point mutation found in the sickle cell β-globin allele (11) as well as detection of a deletion associated with cystic fibrosis (1). The method exploits the sensitivity of PCR amplification to proper base pairing at the 3′ end of the primer (6). We have simplified and extended this procedure for rapid and simple screening of single-base mutants using either PCR fragments, chimeric plasmids or single bacterial colonies. A demonstration of the method and a brief discussion of similar procedures follows.

MATERIALS AND METHODS

The pBluescript® II SK(+), *E. coli* strain JM109 and Perfect Match™ were obtained from Stratagene (La Jolla, CA). *Taq* DNA Polymerase, deoxynucleotides and PCR buffer were obtained from Perkin-Elmer Cetus (Norwalk, CT). Oligonucleotides were synthesized using a MilliGen/Biosearch 8700 DNA Synthesizer (Burlington, MA). The oligos were cleaved from the resin using standard procedures and purified by threefold extraction with an equal volume of *n*-butanol followed by desiccation and resuspension in sterile, distilled water.

E. coli strain JM109 colonies containing pBluescript II SK(+) were either sampled directly for PCR or standard plasmid isolation from bacterial colonies was performed as follows: One medium-size colony was subjected to vigorous vortexing in 10 µl of sterile, distilled water and boiled in a water bath for 10 min. The cellular debris was removed by centrifugation at room temperature for 5 min in a lab-top microcentrifuge. PCRs (50 µl final volume) contained 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 200 µM of each of the four deoxynucleoside triphosphates (dNTPs), 1 µM of each primer, 1–5 ng of mini-prep template DNA or 10 µl of clarified bacterial colony lysate as described, 0.25 units of Perfect Match and 1.25 units of *Taq* DNA polymerase. PCR was routinely performed for 30 cycles in a DNA Thermal Cycler (Perkin-Elmer Cetus) with 1-min denaturation at 94°C

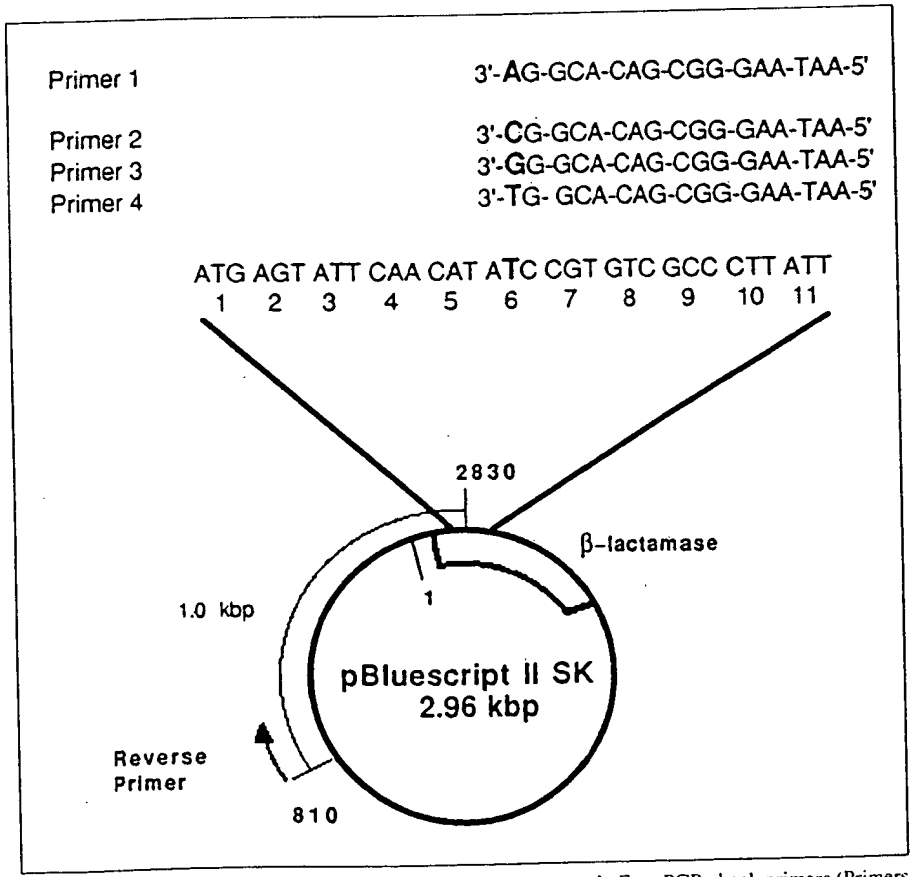

Figure 1. Schematic of the PCR mutagenesis screening approach. Four PCR check primers (Primers 1–4) were designed to complement the region corresponding to β-lactamase codons 6–11. Only Primer 1 was fully complementary. Primers 2–4 contained unique 3′-terminal mismatches. Relevant terminal nucleotides are shown in bold face. Primers 1–4 were each used with the M13 reverse primer, which is complementary to a portion of the pBluescript SK(+) polylinker. The target sequences of the reverse primer and PCR screening primers are indicated using the standard pBluescript II SK(+) numbering. The expected amplification product was 1.0 kb.

and 1 min annealing at 45°C followed by a 2-min extension at 72°C.

RESULTS AND DISCUSSION

The oligonucleotides described in Figure 1 were used to determine how well a series of four primers with unique 3′ termini could distinguish the second base of codon 6 in the β-lactamase gene of pBluescript II SK(+). As shown in Figure 2, only primer 1, which is complementary to the β-lactamase template, acted as an efficient primer for amplification under the conditions described using either bacterial lysate (Figure 2, lane A) or mini-prep template DNA (Figure 2, lane E). Primers with C, G or T 3′ termini, respectively, failed to amplify the 1.0-kbp fragment using either bacterial lysate (Figure 2, lanes B–D) or mini-prep template DNA (Figure 2, lanes F–H). These amplifications were performed multiple times using different stock solutions. These results confirm the reports of Wu and colleagues (11) that the 3′ terminus is critical to efficient PCR. Moreover, the present modifications extend the ease of this screening approach in two ways. First, we have adapted this technique for either mini-prep DNA or bacterial cell lysate. The sensitivity and rapidity of this method is noteworthy since one can get single-base discrimination using unpurified template. Using the colony lysate approach, the entire procedure from colonies on the agar plate takes no more than 3.5 h or less depending on the length of the amplified diagnostic fragment. Second, in our experience, the PCR conditions described were generally applicable to all diagnostic PCR amplifications tested. To this end we found that the inclusion of 0.25 units of Perfect Match per reaction obviated the need for empirical and potentially time-consuming determinations of individual optimal annealing temperatures and optimal magnesium, dNTP or *Taq* DNA polymerase concentrations to achieve good primer discrimination. Moreover, the inclusion of Perfect Match eliminated spurious bands (data not shown). Although we have not tested other PCR-enhancing reagents for their ability to reproduce this effect, we are unaware of other reported instances where such reagents have provided similar enhancement of PCR-based, single-nucleotide discrimination. With ASPCR, Wu and coworkers (11) tested terminal A:A and T:T mismatches. They found that these mismatches were not extended at 55°C but were extended at annealing temperatures of 44°C and 50°C. However, under different conditions Kwok and coworkers (6) found that the presence of one or two T nucleotides at the 3′ terminus of the primer allowed efficient amplification at 55°C irrespective of the corresponding nucleotides in the template. Their generalization was that pyrimidine-pyrimidine and purine-pyrimidine 3′ primer mismatches allow sufficient annealing for subsequent PCR extension, whereas purine-purine mismatches, in general, prevent extension. These generalizations, however, appear to depend heavily on buffer and other PCR conditions. For example, Kwok and colleagues (6) observed that decreasing the dNTP level to 6 μM gave better discrimination for 3′ T mismatches, excluding the T:G mismatch. In contrast, the present results indicate that all three possible terminal T mismatches can be equally discriminated under standard PCR conditions, especially when using mini-prep DNA. Even bacterial colony lysates showed clear negative results with all three 3′-terminal mismatches; however, T:T mismatches gave some extra minor bands. Primers with all four possible 3′ termini were tested against a T nucleotide within a plasmid template. Although we haven't tested similar PCR screens against other template nucleotides, it seems reasonable that equally good or better discrimination is possible with other nucleotides, especially since primers with 3′-terminal T mismatches have historically been the most difficult to resolve using ASPCR. The present results indicate that, contrary to Wu and colleagues (11), it is unnecessary to avoid certain 3′ mismatches, such as T mismatches, when using this screening method.

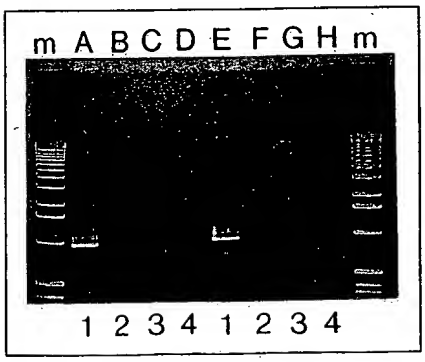

Figure 2. PCR screening results. Ethidium bromide-staining 0.75% agarose gel. PCR amplifications were performed as described in the text using bacterial lysate (lanes A–D) and mini-prep pBlueScript II SK(+) plasmid DNA (lanes E–H). Primers used are shown at the bottom. 1-kb ladder (GIBCO BRL/Life Technologies, Gaithersburg, MD) is shown flanking lanes A and H.

To screen colonies, a minimum of one unique primer is required. We commonly utilize a screening primer whose 3′-terminal base is complementary to the mutant base(s). One can use the same primer with the wild-type template as a negative control to rule out any spurious PCR amplification products. It may also be prudent to include a positive control. One could synthesize a primer complementary to the wild-type template to check for the expected amplification product. The cost of this method is one or two 17-mer oligonucleotide primers. As the cost of oligonucleotide synthesis and reagents continues to decrease, this kind of screening assay will become more attractive. In conclusion, this modified method appears easy, cost-efficient and generally applicable to many forms of mutations, with the convenience of PCR.

REFERENCES

1. **Ballabio, A., R.A. Gibbs and C.T. Caskey.** 1990. PCR test for cystic fibrosis. Nature *343*:220.
2. **Clackson, R. and G. Winter.** 1989. 'Sticky feet'-directed mutagenesis and its application to swapping antibody domains. Nucleic Acids Res. *17*:10163-10170.
3. **Higuchi, R., G. Krummel and R.K. Saiki.** 1988. A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. *16*:7351-7367.
4. **Ho, S.N., H.D. Hunt, R.M. Horton, J.K. Pullen and L.R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene *77*:51-59.
5. **Kuppuswamy, M.N., J.W. Hoffmann, C.K. Kasper, S.G. Spitzer, S.L. Groce and S.P. Bajaj.** 1991. Single nucleotide primer extension to detect genetic diseases: Experimental application to hemophilia B (factor IX) and

cystic fibrosis genes. Proc. Natl. Acad. Sci. USA *88*:1143-1147.

6. **Kwok, S., D.E. Kellogg, N. McKinney, D. Spasic, L. Goda, D. Levenson and J.J. Sninsky.** 1990. Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies. Nucleic Acids Res. *18*:999-1005.
7. **Landegren, U., R. Kaiser, J. Sanders and L. Hood.** 1988. A ligase mediated gene detection technique. Science *241*:1077-1080.
8. **Newton, C.R., A. Graham, L.E. Heptinstall, S.J. Powell, C. Summers, N. Kalsheker, J.C. Smith and A.F. Markham.** 1989. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res. *17*:2503-2516.
9. **Sarkar, G. and S.S. Sommer.** 1990. The "mega-primer" method of site-directed mutagenesis. BioTechniques *8*:404-407.
10. **Sommer, S.S., J.D. Cassady, J.L. Sobell and C.D.K. Bottema.** 1989. A novel method for detecting point mutations or polymorphisms and its application to population screening for carriers of phenylketonuria. Mayo Clinic Proc. *64*:1361-1372.
11. **Wu, D.Y., L. Ugozzoli, B.K. Pal and R.B. Wallace.** 1989. Allele-specific enzymatic amplification of β-globin genomic DNA for diagnosis of sickle cell anemia. Proc. Natl. Acad. Sci. USA *86*:2757-2760.

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Using PCR to Extend the Limit of Oligonucleotide Synthesis

ABSTRACT

A method has been developed to allow one to extend the practical limit of oligonucleotide synthesis by coupling the synthesis reaction to a subsequent PCR. Given that DNA synthesizers are capable of producing reasonable yields of oligonucleotides that are 125–150 bases in length, this method could be used to recover the minute amount of full-length product present in mixtures extended well beyond the established limits. This technology could be applied to gene synthesis and mutagenesis.

INTRODUCTION

Improvements in the technology surrounding the production of synthetic oligonucleotides has led to increased yields, shortened reaction times and improved maximum lengths. For a number of years, however, the maximum length achievable with reasonable yield has stayed at about 125–150 bases. With the advent of the PCR technique, one should be able to extend the length of synthetic oligonucleotides well beyond the traditional limits because the percent yield of full-length oligonucleotide need no longer be a primary concern (2).

In the method reported here, we amplified out a full-length copy of a 110-mer oligonucleotide from a particularly poor synthetic mixture where >99% of the nucleotides in the mixture were much shorter than 110 bases. We were able to obtain the full-length copy by amplifying either from the crude synthesis mixture or from a gel purified sample.

MATERIALS AND METHODS

PCR amplification was performed by adding 2 µl 10× PCR buffer (10×: 500 mM KCl, 500 mM Tris-HCl, pH 8.4, 15 mM $MgCl_2$, 1 mg/ml bovine serum albumin [BSA]), 20 pmol of each PCR primer, 2 µl 2 mM deoxynucleoside triphosphates (dNTPs) 0.5 units *Taq* DNA Polymerase (Perkin-Elmer Cetus, Norwalk, CT) to 50 ng of unpurified synthetic 110-mer in a total volume of 20 µl. Gel purified 110-mer was obtained by running the crude mixture and on a 6% denaturing (7 M urea) polyacrylamide gel along with a size standard. The region correspond-

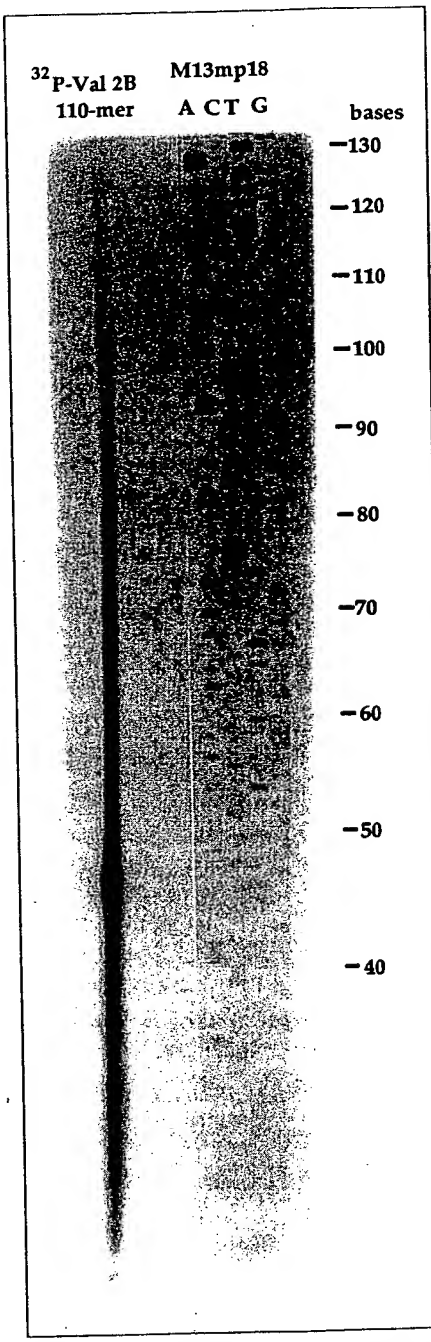

Figure 1. Analysis of the 110-mer crude synthetic mix. The crude synthetic mixture was ^{32}P-labeled and electrophoresed on a 6% denaturing polyacrylamide gel along with an M13mp18 sequencing ladder as a size standard. The amount of 110-mer product was estimated by measuring transmittance of the autoradiogram with a Bio-Rad 620 Video Densitometer (Richmond, CA).

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Elimination of background signals in a modified polymerase chain reaction-based reverse transcriptase assay

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Abstract

Three highly sensitive reverse transcriptase (RT) assays were recently published that are at least one million times more sensitive than conventional RT assays. These assays derive their high sensitivities through the ability to amplify the complementary DNA (cDNA) product of the RT reaction by the polymerase chain reaction (PCR). We describe a modified PCR-based RT (PBRT) assay that retains the high sensitivities of the original assays while reducing their inherent background signals. The background signal of the PBRT assay was found to be due to an intrinsic RNA-dependent DNA polymerase activity of the Taq DNA polymerase, the enzyme used for the PCR. It could be eliminated by inserting a ribonuclease digestion step prior to amplifying the cDNA product of the RT reaction by PCR and by using a thermostable DNA polymerase identified as having reduced RNA-dependent DNA polymerase activity. Comparable results were obtained using three RNA templates with two purified RT enzymes. This modified assay is capable of detecting reliably between 10 and 100 molecules of RT, which is equivalent to between 1 and 10 retrovirus particles. © 1997 Elsevier Science B.V.

Keywords: Reverse transcriptase assay; Polymerase chain reaction; Retrovirus detection

1. Introduction

Reverse transcriptase (RT) assays can be used to detect the presence of retroviruses for which the sequence of the viral genome is unknown,

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since they rely on the enzymatic activity of RT to copy an exogenous RNA template into a complementary DNA (cDNA) product. Because conventional RT assays, which measure the direct incorporation of a labelled deoxynucleotide opposite an RNA template, usually a homoribopolymer, are not sensitive enough to detect the presence of low levels of retroviruses, a reliable and more sensitive RT assay could increase the power of detection. Such assays have the potential:

1. to identify novel retroviruses,
2. to reveal the presence of known or unknown retroviruses in patients with diseases for which a viral aetiology is suspected but for which no retrovirus has been detected or isolated; and
3. to detect low levels of contaminating retroviruses in vaccines and other biological products for medical or veterinary use.

Three very sensitive RT assays that are approximately one-million times more sensitive than conventional RT assays have been described recently (Heneine et al., 1995; Pyra et al., 1994; Silver et al., 1993). All three methods rely on the same strategy: an RT reaction is carried out with an RNA template of known sequence and an oligodeoxynucleotide primer; this is followed by the amplification of the resulting cDNA by the polymerase chain reaction (PCR), and the PCR product is then detected by one of several techniques. These PCR-based RT (PBRT) assays have been referred to as PERT (product-enhanced RT; Pyra et al., 1994) or Amp-RT (Heneine et al., 1995; Yamamoto et al., 1996) assays. The high sensitivity of these PBRT assays is due to the ability to amplify the cDNA product by the PCR. When using purified RT enzymes of known specific activity, these assays have been reported capable of detecting the equivalent of fewer than 10 virions (Pyra et al., 1994; Silver et al., 1993), assuming the presence of between 20 and 100 molecules of RT per virion (Bauer and Temin, 1980; Kacian et al., 1971; Krakower et al., 1977; Layne et al., 1992; Panet et al., 1975; Stromberg et al., 1974). With such sensitivity, the PBRT assays are obviously susceptible to the problems common with all sensitive PCR detection methods, such as the detection of low levels of contaminating DNA. To avoid some of the sources of potential contamination, templates for two of the methods were chosen to be from the genome of RNA viruses that had no DNA in their life cycles and whose sequences had little homology with mammalian genomes: the plant virus brome mosaic virus (BMV) by Silver et al. (1993) and the bacteriophage MS2 by Pyra et al. (1994). In the case of the Amp-RT method, Heneine et al. (1995) cloned a segment of the genome of encephalomyocarditis virus (EMCV) into an RNA expression vector, and this plasmid was used to produce the RNA template by transcription in vitro. The original plasmid DNA was degraded subsequently by two consecutive treatments with DNase to produce the RNA free of DNA. A potential problem with this last method is that any small amounts of residual DNA may be amplified during the PCR step, although the authors assay for this and claim that it is not a problem.

Since it was possible that retroviral RT's would have varying activities on different RNA templates, which would give rise to signals in the PBRT assays that were not accurate reflections of the number of retrovirus particles, it was necessary to compare the assays using several purified RT's and retroviruses. Because we found that their sensitivities varied and they had background signals when buffer was used as a sample, it was decided to characterise the reaction in an attempt both to optimise the assays and to determine the cause of the background signals. We describe a modified PBRT assay protocol that is equally sensitive using three commercially-available RNA templates. We report that the background signal of the assay is due to an intrinsic RNA-dependent DNA polymerase activity of the AmpliTaq DNA polymerase and that this signal can be eliminated by treating the products of the RT step with RNase prior to the PCR step and by using other thermostable DNA polymerases that we identified as having reduced attendant RNA-dependent DNA polymerase activities.

Table 1
Sequences

Name
B1
B2
B3
B4
M1
M2
M4
T1
T2
T3
T4

2. Mate

2.1. RN
and olig

Brom
chased
number
virus (
Mannhe
165 948
myelobl
(RT) w
number
leukaem
28 025-(
logue n
nologies
free) wa
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0138), a
Elmer
NJ); clo
were fr
and Ver
labs (Be
Boehrin
Oligo
Facility

Table 1
Sequences and functions of oligodeoxynucleotides

Name	Genome	Purpose	Sequence
B1	BMV	RT primer	GGT CTC TTT TAG AGA TTT ACA GTG
B2	BMV	Upstream PCR primer	CGT GGT TGA CAC GCA GAC CTC TTA C
B3	BMV	Downstream PCR primer	TCA ACA CTG TAC GGC ACC CGC ATT C
B4	BMV	PCR product probe	GCC TTT GAG AGT TAC TCT TTG
M1	MS2	RT primer, PCR primer	CAT AGG TCA AAC CTC CTA GGA ATG
M2	MS2	Upstream PCR primer	TCC TGC TCA ACT TCC TGT CGA G
M4	MS2	PCR product probe	TTA ATG TCT TTA GCG AGA CGC
T1	TMV	RT primer	AGT GTC GAA TGC ACC TAA CAG TGC
T2	TMV	Upstream PCR primer	GCC TTA GGA AAT CAG TTT CAA ACA C
T3	TMV	Downstream PCR primer	CTA GCG GGT CTA ATA CCG CAT T
T4	TMV	PCR product probe	CGA ACT GTC GTT CAA AGA CAA TTC

2. Materials and methods

2.1. RNA templates, enzymes, RNase inhibitor, and oligodeoxynucleotides

Brome mosaic virus (BMV) RNA was purchased from Promega (Madison, WI; catalogue number D1541), while MS2 and tobacco mosaic virus (TMV) RNA's were from Boehringer-Mannheim (Indianapolis, IN; catalogue numbers 165948 and 1120387, respectively). Avian myeloblastosis virus (AMV) reverse transcriptase (RT) was obtained from Promega (catalogue number M5101; 10 U/μL), and Moloney murine leukaemia virus (M-MLV) RT (catalogue number 28025-013; 200 U/μL) and T4 DNA kinase (catalogue number 18004-028) were from Life Technologies (Gaithersburg, MD). RNase (DNase free) was purchased from Boehringer-Mannheim (catalogue number 1119-915). RNasin was from Promega (catalogue number N2512).

The following thermostable DNA polymerases were used: AmpliTaq (N808-0171), native Taq (N808-0168), AmpliTaq Stoffel fragment (808-0138), and UlTma (N808-0117) were from Perkin-Elmer (Roche Molecular Systems, Branchburg, NJ); cloned Pfu (600153) and native Pfu (600135) were from Stratagene (La Jolla, CA); Vent (254) and Vent exo-(257) were from New England Biolabs (Beverly, MA); and Pwo (1644947) was from Boehringer-Mannheim.

Oligodeoxynucleotides were synthesised in the Facility for Biotechnology Resources, CBER. The primers for MS2 and BMV have been described (Pyra et al., 1994; Silver et al., 1993). Two sets of primers were chosen for TMV from different regions of the genome. Because Silver et al. (1993) used a specific primer for the RT reaction while Pyra et al. (1994) used the RT primer as the downstream primer in the subsequent PCR step, both sets of primers were designed for use either in the format described by Silver et al. (1993) (i.e., three primers) or the format described by Pyra et al. (1994) (i.e., two primers) in order to be able to compare their efficiencies. A description and sequences of the oligonucleotides used in the PBRT reactions are given in Table 1.

2.2. PCR-based RT (PBRT) assay

In order to avoid contamination, three rooms were used routinely and solutions and supplies were sterilised by autoclaving where possible. Stock solutions were distributed in a laminar flow hood in the first (clean) room; this room was also used to prepare the RT and PCR reaction mixtures on the day of use. The second (assay) room was used for sample preparation (i.e., serial dilutions) and assembly of the complete RT and PCR reactions. In the third (analysis) room, dilutions of the concentrated RT's were carried out, as well as all the analyses of the PCR products.

2.2.1. Reverse transcriptase step

The reaction volume for the RT step was 25 μL of which the sample volume was either 3 μL or

10 μL. A master mixture was prepared as follows. To the appropriate volume of sterile water were added: 10 × RT buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 50 mM $MgCl_2$ and 3.5% Triton X-100), 10 × solution of the four deoxynucleoside triphosphates (dNTP's; 2 mM each), 10 × DTT (20 mM), 10 × oligodeoxynucleotide RT primer (1.3 μM), and RNasin (to 8 units per reaction). At this stage, a volume was removed for the minus RNA controls. RNA template (approximately 0.3 pmol per reaction) was added to the remainder, and the appropriate volume distributed to sterile MicroAmp tubes (Perkin Elmer; catalogue number N801-0540). The final concentrations were 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM each of the four dNTP's, 2 μM DTT, 0.35% Triton X-100, 8 U RNasin, 0.13 μM primer (3.25 pmol), and 0.3 pmol of template RNA; these values do not take into account the amounts contributed by the sample. Samples (10 μL or 3 μL) were added, and the tubes were incubated at 37°C for times varying from 3 h to 5 h.

For every experiment, both positive and negative controls were included. Negative controls were the omission of RNA from the RT reaction, the use of enzyme dilution buffer as a sample, and digestion of the RT components with RNase prior to RT addition. Enzyme dilution buffer is 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.25 mM EDTA, pH 8.0, 0.2 mM DTT, 0.025% Triton X100, and 50% glycerol. For the positive controls, fourteen 10-fold serial dilutions in enzyme dilution buffer of a purified RT (usually AMV, but sometimes M-MLV RT) were prepared (in a laminar flow hood in the analysis room) and stored in a benchtop cooler at −20°C; dilutions eight to 14 were assayed. Fresh dilutions were made for each assay. An additional control included the thermostable DNA polymerase as a sample (to determine its RNA-dependent DNA polymerase activity).

2.2.2. Polymerase chain reaction (PCR)

PCR mixtures were prepared while the RT reactions were incubating. A master mixture was prepared from 10 × stock solutions and consisted of: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 μM each dNTP, 1 μM primer (or 0.87 μM if the downstream primer is the same as that used for the RT step), 2 units RNase, and the thermostable DNA polymerase (2.5 units, or the amount recommended for PCR by the manufacturer). 10 × PCR buffer comprises: 100 mM Tris-HCl, pH 8.3, and 0.5 M KCl. Thus, the final concentrations in the PCR are: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM each dNTP, 1 mM DTT, 0.175% Triton X100, 0.5 μM of each primer, 2 units RNase, and 2.5 units of thermostable DNA polymerase. These concentrations do not take into account those amounts contributed by the sample. In the clean room, complete PCR components (25 μL) were distributed into MicroAmp tubes. In the assay room, RT reactions were transferred to the tubes containing the PCR mixtures; this procedure was chosen to minimize the chance of contamination. Incubations were conducted in the Perkin-Elmer 9600 Thermocycler. RNase digestion was for 30 min at 37°C. PCR conditions were: 94°C for 1 min; 35 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s; and 72°C for 10 min.

2.2.3. Detection methods for the PCR products

2.2.3.1. Agarose gel electrophoresis and ethidium bromide staining. Following the PCR step, 10 μL of the reaction plus 2 μL of 6 × loading buffer (15% Ficoll 400, 60 mM EDTA, pH 7.5, 0.25% bromophenol blue, 0.25% xylene cyanol FF) were analysed by electrophoresis through 1.8% agarose gels in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) in the presence of 0.2 μg/mL ethidium bromide using a BioRad apparatus (Wide Mini-Sub Cell GT model) at 80 volts for about 2 h.

2.2.3.2. Southern hybridisation analysis. After photography, the gels were soaked in 0.4 M NaOH for 30 min, and the DNA was transferred to a charged nylon membrane (Hybond-N +, Amersham) by capillary transfer in 0.4 M NaOH (Reed and Mann, 1985). The transfer was continued for between 24 and 48 h, changing the paper towels when saturated with liquid. Following the transfer, the filters were neutralised by shaking for 30

min at room temperature in 0.4 M Tris-HCl, pH 7.2, 0.8 M NaCl and air-dried on Whatman 3MM paper. Dried filters can be stored at this point. For the pre-hybridisation, filters were incubated in 6 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 0.1% SDS, and 0.5% BLOTTO (non-fat powdered milk; Johnson et al., 1984), about 100 mL per filter, at 55°C with shaking for 90 to 120 min. For the hybridisation, ^{32}P end-labelled labelled oligodeoxynucleotide probe (ca. 5×10^6 cpm per filter, specific activity from 5×10^6 to 5×10^8 cpm/mg) was added to 35 mL of the prehybridisation solution, and up to ten filters were incubated with shaking at 55°C for 16 to 24 h in 500 mL Nalgene utility boxes. After hybridisation, the filters were washed with shaking as follows. First wash: in 200 mL of 6 × SSC, 0.5% SDS (SSC is 0.15 M NaCl, 0.015 M sodium citrate) at 55°C for 1.5 h. Second wash: in 400 mL of 2 × SSC, 0.25% SDS at 55°C for 1.5 h. Third wash: in 200 mL of 1 × SSC, 0.1% SDS at room temperature for 0.5 h. The filters were drained on paper towels, dried, and exposed to Kodak XAR film from 3 to 18 h at room temperature.

2.2.3.3. Cycled primer extension. This was done essentially as described by Silver et al. (1993). Following the PCR, 8 μL of the products were withdrawn to another PCR that contained 2 μL of a solution containing 1 × PCR buffer, AmpliTaq DNA polymerase (2.5 U), and 1×10^5 cpm of a ^{32}P-end-labelled oligonucleotide (0.01 μg; specific activity 10^7 cpm/μg). Amplification in a Perkin-Elmer 9600 machine was as follows: 94°C for 1 min, four cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, then 72°C for 5 min and held at 10°C. Two microlitres of 6 × loading dye were added, and the labelled primer-extended products were fractionated by electrophoresis on 8% polyacrylamide gels. The gels were dried and exposed to autoradiography with Kodak XAR film at room temperature for between 3 and 18 h. For quantification, the autoradiographs were used as templates to excise the radioactive bands from the dried gels. Each piece was counted in a liquid scintillation spectrometer (Beckman LS5801) in CytoScint (ICN, Costa Mesa, CA; catalogue number 882453).

2.3. End-labelling of oligonucleotides

Oligonucleotides (800 ng) were labelled using T4 DNA kinase (Life Technologies, Gaithersburg, MD) and 250 μCi of γ[^{32}P]ATP (Amersham Life Sciences, Inc., Arlington Heights, IL) according to the manufacturer's forward reaction protocol. The reaction was stopped by addition of EDTA to 20 mM, and the labelled oligonucleotide was purified by ethanol precipitation with ammonium acetate (to 2.5 M), $MgCl_2$ (to 40 μM), yeast tRNA to 5 μg/mL, and three volumes of ethanol.

2.4. Polyacrylamide Gel Electrophoresis (PAGE)

Eight percent acrylamide gels in 0.5 × TBE buffer were prepared using a Protogel gel mix (30% acrylamide, 0.8% bisacrylamide; National Diagnostics, Atlanta, GA) for the BRL vertical gel apparatus (model V-16 or V-16-2) with 1.5 mm spacers and 20-slot combs. Electrophoresis was for 12 to 14 h at 30–35 volts and 10–12 mA.

2.5. Preparation of cell extracts

The sources of H9 and HeLa cells have been described (Peden et al., 1991). H9 cells were grown in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) and 2 mM glutamine, while the HeLa cells were carried in DMEM with 10% FBS and 2 mM glutamine. To prepare cell extracts, 10^7 cells were collected by centrifugation (1000 rpm for 5 min in a Sorvall RT6000B) and suspended in 0.5 mL of phosphate buffered saline. The cells were lysed by addition of 0.5 mL of enzyme dilution buffer containing 1 μg/mL aprotinin and 0.7 mg/mL pepstatin on ice and freezing at −80°C. Extracts were thawed at 37°C for 15 min and snap frozen at −80°C. The extracts were thawed at 37°C for 15 min and clarified by centrifugation in a Tomy centrifuge (model MTX-152) at 14 000 rpm at 4°C for 1 min. Lysates were distributed into 1.5 mL screw cap polypropylene tubes and stored at −80°C.

3. Results

Although the three published PCR-based RT (PBRT) assays all rely on the same principle, they have several differences that are likely to affect the sensitivity of the reaction and may be important for the specificities of the particular reverse transcriptase (RT) to be detected. These assays are summarised in Table 2. Because both the MS2 and BMV RNA genomes are commercially available, we have concentrated on the Silver-Repaske and PERT assays. We point out some of the obvious differences between these two assays.

1. Two primers are used in the PERT assay, whereas three primers are used in the Silver-Repaske assay; in the PERT assay, the downstream PCR primer is the same as the one used in the RT reaction, while a separate one is used as the downstream PCR primer in the Silver-Repaske assay.
2. The amount of RNA template for the RT reaction in the PERT assay is about 10 times more than that used in the Silver-Repaske assay.
3. The ratio of the RT primer to template is 32:1 for the PERT assay and 12:1 for the Silver-Repaske assay.
4. The PERT assay uses Triton X100 as the non-ionic detergent, whereas the Silver-Repaske assay has NP40.
5. The PERT assay incubates the RT step for 5 h, whereas the Silver-Repaske assay incubation time is 1 h.
6. The PERT assay incorporates an RNase incubation prior to the PCR step.
7. The number of PCR cycles differs, with Silver-Repaske assay using 40 and the PERT assay using 25 cycles.
8. The methods used for the detection of the PCR product differ.

First, we compared the sensitivity of the methods used for the detection of the PCR product. Three methods were tested: agarose gel electrophoresis of the PCR product and staining with ethidium bromide, Southern hybridisation analysis of that gel, and a cycled primer extension reaction followed by polyacrylamide gel electrophoresis. This last procedure incorporates a radioactive label into the PCR product by using a specific internal ^{32}P-end-labelled oligonucleotide and four cycles of PCR amplification (Silver et al., 1993). A comparison of the three methods of detection of the PCR product from a PBRT reaction using MS2 RNA as template and dilutions of avian myeloblastosis virus (AMV) RT is shown in Fig. 1. Ethidium bromide staining (Fig. 1A) was able to detect dilutions of AMV RT down to 10^{-10} (which corresponds to 10^{-8} units), whereas the Southern (Fig. 1B) and cycled primer extension (Fig. 1C) analyses could detect the AMV RT dilution of 10^{-11} (10^{-9} units). Therefore, there was about a log increase in sensitivity when either Southern or cycled primer extension was used over ethidium bromide staining. The heterogeneity of the bands seen in the cycled primer extension analysis was a consequence of the various single-stranded and partially double-stranded forms that would be present. This was confirmed by analysing the cycled primer extended products on denaturing gels, where a single band was found (data not shown). Although in this particular assay the Southern and cycled primer extension analyses had comparable sensitivities, we found that the latter method is usually slightly more sensitive, and because in our hands it was found to be more reproducible, this method was used for most of our analyses.

When we compared the three published assays, the PERT assay was between 10 and 50 times more sensitive than the Silver-Repaske assay and 100 to 1000 times more sensitive than the Amp-RT assay. Minor variations in the concentrations of the buffer components and dNTP's had little effect on the overall sensitivity of the PBRT reaction. The presence of RNasin in the RT step had little effect when purified enzymes were used. However, inclusion of this RNase inhibitor was important when crude samples were analysed (see below). Variables that had a significant effect were: the presence of RNase when using the AmpliTaq DNA polymerase (see below); the amounts of RNA template and primer for the RT step (see below); the time of incubation of the RT step; and the number of PCR cycles. Because we found that the sensitivity of the reaction increased with increasing time of the RT reaction, incubation times

Table 2
Differenc

Reagent/

RT Step
Sample v
RT volu
Final vol
RNA ten
Amount

Primer a

RNasin
Buffer co
Tris-HCl
KCl
$MgCl_2$
dNTP's
DTT
NP40
Triton X
BSA
EGTA
Time of

PCR ste
PCR vol
Final vo
Amount
5′ Prime
3′ Prime

Final cor
5′ Prime
3′ Prime
Taq DN
UlTma I
DNA
Buffer c
Tris-HC
Final
KCl
Final
$MgCl_2$
Final
dNTP's
Final
Gelatin
Final
RNaseA
Number

Table 2
Differences among the various PBRT assays

Reagent/condition	Assay			
	Silver-repaske	PERT	Amp-RT	PBRT
RT Step				
Sample volume	10 μL	3 μL	10 μL	10 μL (or 3 μL)
RT volume	15 μL	25 μL	40 μL	15 μL (or 22 μL)
Final volume	25 μL	28 μL	50 μL	25 μL
RNA template	BMV	MS2	EMCV	BMV, MS2
Amount	21 ng (0.021 pmol)	300 ng (0.28 pmol)	10 ng (0.087 pmol)	280 ng (0.28 pmol), 300 ng (0.28 pmol)
Primer amount	10 nM (0.25 pmol)	72 ng (9 pmol)	100 ng (12.6 pmol)	133 nM (3.33 pmol; 9 pmol)
RNasin	8 U	25 U	10 U	8 U
Buffer components				
Tris-HCl, pH 8.3	10 mM	50 mM	50 mM	10 mM
KCl	50 mM	50 mM	50 mM	50 mM
$MgCl_2$	5 mM	8 mM	10 mM	5 mM
dNTP's	0.1 mM	0.893 mM	0.4 mM	0.2 mM
DTT	2 mM	10 mM	2 mM	2 mM
NP40	0.1%	–	0.06%	–
Triton X100	–	0.357%	–	0.35%
BSA	–	0.115 mg/mL	–	–
EGTA	–	–	0.8 mM	–
Time of Incubation	1 h	5 h	2 h	3–5 h
PCR step				
PCR volume	25 μL	75 μL	50 μL	25 μL
Final volume	50 μL	103 μL	100 μL	50 μL
Amount of primers				
5' Primer	1 μM	25 pmol	6.18 pmol	1 μM (25 pmol)
3' Primer	1 μM	14 pmol	–	0.87 μM (21.75 pmol) or 1 μM (25 pmol)
Final concentrations				
5' Primer	0.5 μM	0.25 μM	0.06 μM	0.5 μM (25 pmol)
3' Primer	0.5 μM	0.25 μM	0.13 μM	0.5 μM (25 pmol)
Taq DNA polymerase	2.5 U	2.5 U	2.5 U	–
UlTma DNA polymerase (or Pfu DNA Polymerase)	–	–	–	2.5 U
Buffer components				
Tris-HCl, pH 8.3	10 mM	10 mM	10 mM	10 mM
Final	10 mM	37.5 mM	30 mM	10 mM
KCl	50 mM	37.5 mM	100 mM	50 mM
Final	50 mM	40.6 mM	75 mM	50 mM
$MgCl_2$	–	–	–	–
Final	2.5 mM	4 mM	5 mM	2.5 mM
dNTP's	0.2 mM	–	0.4 mM	0.2 mM
Final	0.15 mM	0.243 mM	0.4 mM	0.2 mM
Gelatin	–	0.01%	–	–
Final	–	0.001%	–	–
RNaseA	–	8 ng	–	500 ng
Number of cycles	40	25	35	35

from 3 to 5 h at 37°C were used. Also, the sensitivity of the reaction was increased by raising the number of PCR cycles from 25 to 35.

In order to have two assays with comparable sensitivities, we decided to modify the Silver-Repaske assay, because BMV RNA is commercially available. The original amounts of RT primer and template in the Silver-Repaske assay were 0.25 pmol and 0.021 pmol, respectively, which correspond to a primer to template ratio of 12:1. The amount of template RNA was raised to 0.28 pmol, which approximated that used by Pyra et al. (1994) for the PERT assay, and the RT primer was raised to 3.33 pmol, which retains the 12:1 primer to template ratio. With the original lower amounts of RT primer and template, dilutions of AMV RT to 10^{-9} (10^{-7} units) could be detected (Fig. 2A), whereas with the higher amounts, dilutions down to 10^{-10} and even 10^{-11} (10^{-8} units and 10^{-9} units, respectively) could be detected (Fig. 2B). Thus, at least a 10-fold increase in sensitivity was achieved. This sensitivity was equivalent to that found with MS2 RNA as template (compare with Fig. 1). Increasing the ratio of RT primer to template to 32:1, which was the ratio used by Pyra et al. (1994), did not result in an additional increase in sensitivity. While there was no signal in the negative control reactions (Fig. 2, lanes 1, 2, 8, and 9), increasing the sensitivity of the reaction had the consequence that a signal became apparent when the AmpliTaq DNA polymerase was used as a sample (Fig. 2B, lane 3). This was also found using MS2 RNA, in agreement with Pyra et al. (1994), and confirms that the AmpliTaq DNA polymerase has an RNA-dependent DNA polymerase activity (Jones and Foulkes, 1989).

The RNA-dependent DNA polymerase activity of the AmpliTaq DNA polymerase could be due either to a contaminating enzyme or to an intrinsic enzymatic activity. We compared the RT activity of native Taq DNA polymerase, which is

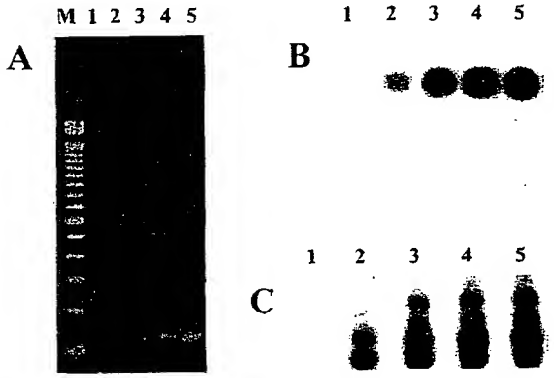

Fig. 1. Comparison of the sensitivities for the various detection methods of the PCR product. The RT reaction was carried out with MS2 RNA as template and dilutions of AMV RT as enzyme for 5 h at 37°C. The cDNA product of the RT reaction was amplified using AmpliTaq DNA polymerase as described in Section 2, and the PCR product was analysed by three methods: (A) Agarose gel electrophoresis and ethidium bromide staining, (B) Southern analysis, and (C) cycled primer extension. Lane M: 100 bp ladder. Lanes 1–5: 10-fold dilutions of AMV RT between 10^{-12} and 10^{-8}, respectively.

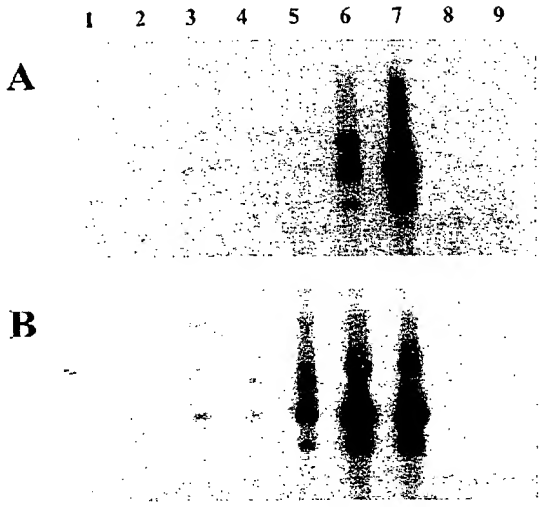

Fig. 2. Modification of the Silver-Repaske PBRT assay. (A) The original amounts of BMV RNA template and RT primer (Oligo B1) were used (0.021 pmol and 0.25 pmol, respectively). (B) The template and RT primer amounts were increased to that used in the PERT assay with MS2 RNA as template, adjusting for the size of the MS2 and BMV genomes (0.28 pmol and 3.33 pmol, respectively). RT incubation was for 3 h at 37°C with the following samples. Lane 1: minus RNA; lane 2: enzyme dilution buffer; lane 3: AmpliTaq DNA polymerase (2.5 U); lane 4: 10^{-11} dilution of AMV RT; lane 5: 10^{-10} dilution of AMV RT; lane 6: 10^{-9} dilution of AMV RT; lane 7: 10^{-8} dilution of AMV RT; lane 8: 10^{-8} dilution of AMV RT incubated with RNase prior to RT incubation; lane 9: enzyme dilution buffer. The cDNA product was amplified using Oligo B3 and Oligo B2 for 35 cycles. The PCR product was subjected to cycled primer extension with end-labelled Oligo B4 and analysed by polyacrylamide gel electrophoresis on 8% gels.

prepared from *Thermus aquaticus*, with the same enzyme expressed in and prepared from *E. coli*, since a co-purifying contaminating enzyme would unlikely be the same in the different organisms. Also, because the native Taq DNA polymerase was prepared from a thermophilic bacterium and therefore any contaminating protein would likely be thermostable, we tested the heat lability of the RNA-dependent DNA polymerase activity of AmpliTaq DNA polymerase. Both the native and cloned DNA polymerases had RNA-dependent DNA polymerase activity, and heating failed to eliminate this activity from either product. These results are consistent with the Taq DNA polymerase having an intrinsic RNA-dependent DNA polymerase activity.

To determine whether other thermostable DNA polymerases also possess an associated RNA-dependent DNA polymerase activity, we tested the AmpliTaq, native Taq, the Stoffel fragment of AmpliTaq, UlTma, native Pfu, cloned Pfu, Pwo, Vent, and Vent exo- DNA polymerases. Using MS2 RNA as template, the nine thermostable DNA polymerases were incubated in the RT reaction for 4 h at 37°C. Following RNase digestion, the products of the RT reactions were all amplified using AmpliTaq DNA polymerase, and the PCR products were detected by cycled primer extension and PAGE. Of all the enzymes tested, the two with the most RT activity were AmpliTaq and native Taq DNA polymerases (Fig. 3; lanes 3 and 4, respectively). The Stoffel fragment of the Taq DNA polymerase, and the UlTma, native Pfu, cloned Pfu, Pwo, Vent, and Vent exo-DNA polymerases had significantly lower activity (Fig. 3; lanes 5–11, respectively).

Eight of these thermostable DNA polymerases (native Taq DNA polymerase was omitted) were tested in a complete PBRT reaction with AMV RT (10^{-7} units) as the RT enzyme. Most functioned well, with the UlTma and native Pfu DNA polymerases performing slightly better than the others (data not shown). These two enzymes were investigated further.

It was possible that the different RNA-dependent DNA polymerase activities of the DNA polymerases would be manifested to varying degrees on different templates. Therefore, the AmpliTaq, UlTma, and native Pfu DNA polymerases were assayed on both the BMV and MS2 RNA templates. In addition, because we considered it important to have a number of templates that could be used in this assay in case different retroviral RT's were found to exhibit template specificity, oligonucleotide primers were designed for a third RNA, that of the plant virus tobacco mosaic virus (TMV; see Section 2). To allow for the detection of any RNA-dependent DNA polymerase activity of the DNA polymerases during the PCR step, RNase was omitted from this assay. AmpliTaq DNA polymerase had RT activity on all three templates, the MS2, BMV, and TMV RNA's (Fig. 4A, D and G; lane 3). In contrast, the UlTma and native Pfu DNA polymerases had activities that depended upon the particular RNA template. For the UlTma DNA polymerase, lower activity was seen with the MS2 RNA (Fig. 4B; lane 3) compared with either the BMV (Fig. 4E; lane 3) or the TMV templates (Fig. 4H; lane 3).

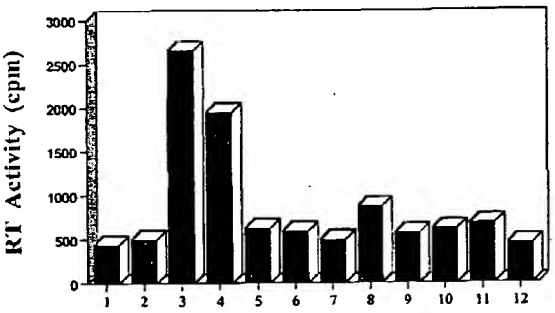

Fig. 3. Analysis of Different Thermostable DNA Polymerases for RT Activity. Each polymerase was used at 2.5 units in the standard reaction buffer for the RT step with MS2 RNA as template and Oligo M1 as primer. Incubations were for 4 h at 37°C. The products of the RT step were all amplified in the PCR reaction by the AmpliTaq DNA polymerase and Oligos M1 and M2. Analysis was by cycled primer extension with Oligo M4 and AmpliTaq DNA polymerase, PAGE, and autoradiography. For the quantification, bands from the gels were excised and counted by liquid scintillation spectrometry. The following were used in the RT reaction. Lane 1: minus RNA; lane 2: enzyme dilution buffer; lane 3: AmpliTaq DNA polymerase; lane 4: native Taq DNA polymerase; lane 5: the Stoffel fragment of AmpliTaq DNA polymerase; lane 6: UlTma DNA polymerase; lane 7: native Pfu DNA polymerase; lane 8: cloned Pfu DNA polymerase; lane 9: Pwo DNA polymerase; lane 10: Vent DNA polymerase; lane 11: Vent exo- DNA polymerase; lane 12: enzyme dilution buffer.

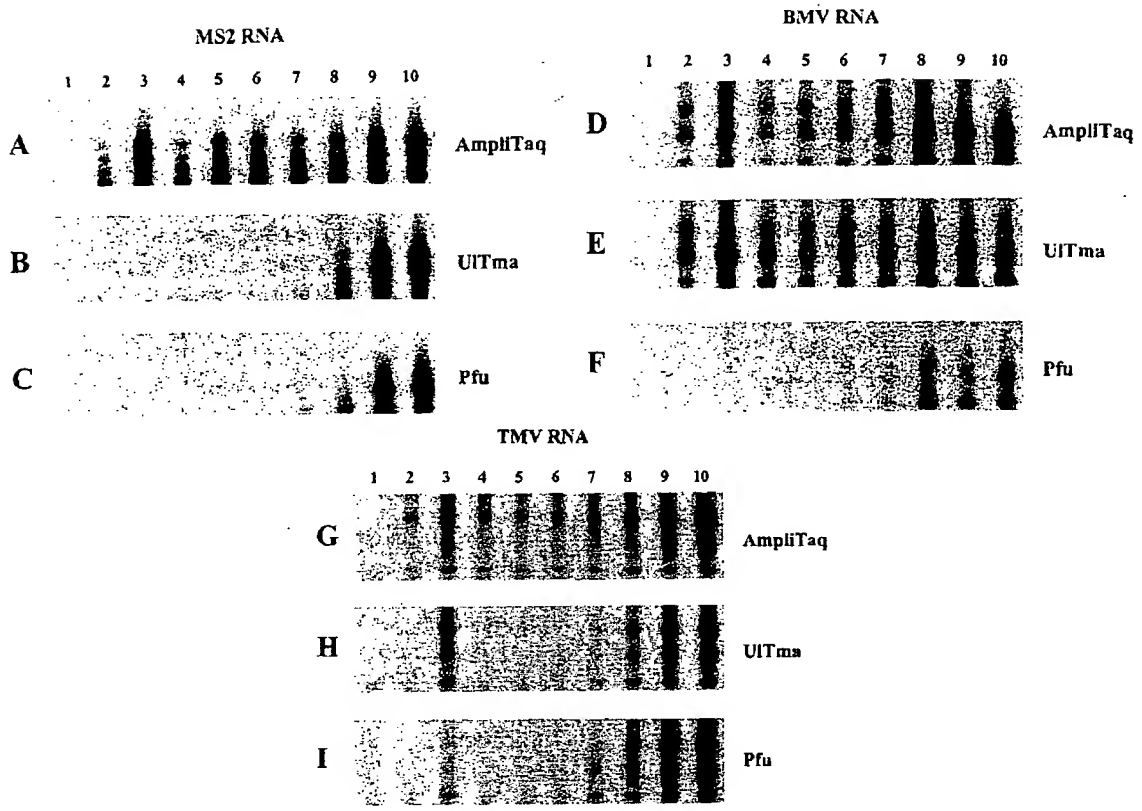

Fig. 4. Activity of AmpliTaq, Native Pfu, and UlTma DNA Polymerases on MS2, BMV, and TMV RNA Templates in the Absence of RNase. Dilutions of AMV RT from 10^{-8} to 10^{-14} were tested on three templates using the three thermostable DNA polymerases to amplify the cDNA reverse transcript. The RT reaction was for 4 h at 37°C. The PCR amplification was as described in Section 2. (A–C) MS2 RNA template, (D–F) BMV RNA template, (G–I) TMV RNA template, (A, D and G) AmpliTaq DNA polymerase, (B, E, H) UlTma DNA polymerase, (C, F, I) native Pfu DNA polymerase. Lane 1: no RNA; lane 2: enzyme dilution buffer; lane 3: thermostable DNA polymerase; lanes 4–10: 10-fold dilutions of AMV RT between 10^{-14} and 10^{-8}, respectively.

For the native Pfu DNA polymerase, low activity was seen with the MS2 RNA (Fig. 4C; lane 3) and BMV RNA (Fig. 4F; lane 3), but this enzyme had activity with TMV RNA (Fig. 4I; lane 3). In summary, all these three thermostable DNA polymerases had RT activities, but this activity was not found with all primer/template combinations.

Not only were there signals when the DNA polymerase was used as a sample, but more importantly signals were found when there was no enzyme present for the RT reaction with some of the polymerases with some of the templates. These signals were due to the thermostable DNA polymerase being able to copy the RNA template during the PCR. With the AmpliTaq DNA polymerase as the PCR enzyme, a signal was produced from all three templates with enzyme dilution buffer as sample (Fig. 4A, D, and G; lane 2). The ability of the AmpliTaq DNA polymerase to copy the RNA during the PCR step most likely accounted for the signals seen in the positive controls with dilutions of the AMV RT down to 10^{-14} and 10^{-13} (Fig. 4A, D, and G; lanes 4 and 5, respectively). Since these dilutions corresponded to approximately 0.8 and 0.08 molecules, respectively, of AMV RT, it was unlikely that the signal was due to the AMV RT but rather due to the RNA-dependent DNA polymerase activity of

the AmpliTaq DNA polymerase. A similar result was found with the UlTma DNA polymerase with the BMV template, where a signal was present both in the buffer controls (Fig. 4E; lane 2) and in the high dilutions of the AMV RT (Fig. 4E; lanes 4 and 5). In the case of the TMV template, the UlTma DNA polymerase was able to copy this RNA if present during the RT incubation step (Fig. 4H; lane 2), but a signal was not present in the 10^{-14} and 10^{-13} dilutions of the AMV RT (Fig. 4H; lanes 4 and 5). Although the Pfu DNA polymerase was able to copy the TMV RNA when used as a sample in the RT incubation step (Fig. 4I; lane 3), no signal was found with this template in the buffer control (Fig. 4I; lane 2) or in the 10^{-14} and 10^{-13} dilutions of the AMV RT (Fig. 4I; lanes 4 and 5).

Thus, several thermostable DNA polymerase and template combinations were identified that produced insignificant background signals while maintaining high sensitivity. The UlTma DNA polymerase with MS2 RNA and the native Pfu DNA polymerase with either MS2 RNA or BMV RNA were capable of detecting dilutions of the AMV RT at least down to 10^{-11}, which corresponds to 10^{-9} units of RT. Using the specific activity of the enzyme provided by the manufacturer, these activities correspond to 83 molecules of the AMV RT.

Because the Silver-Repaske assay used three primers compared with two primers used in the PERT assay, we tested whether a separate primer for the RT affected the sensitivity of the PBRT assay. Primers were designed for use with TMV RNA (see Section 2) so that a two primer assay could be compared with a three primer assay in which the same upstream primer would be used with a separate RT primer or a common RT and downstream PCR primer. Within the variabilities of the assay, no significant differences in the sensitivities were found.

During the course of this work, it was observed that there was lot to lot variation in the assay background signals found with both the UlTma and native Pfu DNA polymerases. This was found to be due to different lots having different amounts of RNA-dependent DNA polymerase activity. Fig. 5 shows the results of a PBRT assay using three lots of native Pfu DNA polymerase as the sample (lanes 2-4) with the BMV RNA as template together with the UlTma (lane 5) and AmpliTaq (lane 6) DNA polymerases. Because of this variability, it was necessary to include an RNase digestion for 30 min at 37°C prior to PCR amplification (see Section 2).

While the presence of the RNase inhibitor RNasin did not influence the sensitivity of the PBRT reaction when purified AMV RT was used as the sample (data not shown), when lysates from human cells were tested the presence of RNasin increased the sensitivity of the PBRT reaction, presumably because of the presence of RNase in the cell extracts. Lysates were prepared from H9 cells as described in Section 2, and different amounts of RNasin were added to a 10^{-2}-dilution of the extracts. Dilution of the extract was necessary because inhibition was found when higher concentrations were used (data not shown). The PBRT signal was lower when no RNasin was present (Fig. 6, lane 1) compared with when it was present (Fig. 6; lanes 2–5). With this extract, amounts from 8 U to 120 U had equivalent results. Similar results were found with extracts from HeLa cells (data not shown). Because it was anticipated that the PBRT assay

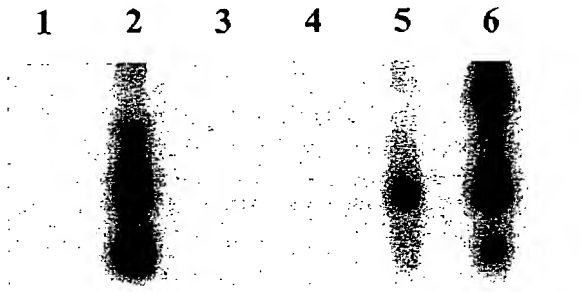

Fig. 5. RNA-dependent DNA polymerase activity of different lots of the native Pfu DNA polymerase. Three lots of the native Pfu DNA polymerase and single lots of the UlTma and AmpliTaq DNA polymerases were incubated with BMV RNA at 37°C for 3 h. The cDNA product was amplified using Pfu DNA polymerase (lot C), and the PCR products were analysed using cycled primer extension and PAGE. Lane 1: enzyme dilution buffer; lane 2: Pfu DNA polymerase lot A; lane 3: Pfu DNA polymerase lot B; lane 4: Pfu DNA polymerase lot C; lane 5: UlTma DNA polymerase; lane 6: AmpliTaq DNA polymerase.

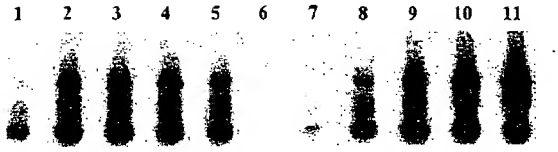

Fig. 6. Effect of RNasin on the PBRT assay with cell extracts. A 10^{-2}-dilution of an extract from H9 cells was incubated with MS2 RNA in various amounts of RNasin for 3 h at 37°C. Positive controls were dilutions of AMV RT and contained the usual 8 U units of RNasin. The cDNA products were amplified using UlTma DNA polymerase and analysed by cycled primer extension and PAGE. Lane 1: 0 U RNasin; lane 2: 8 U RNasin; lane 3: 20 U; lane 4: 40 U RNasin; lane 5: 120 U RNasin; lanes 6–11: 10-fold dilutions of AMV RT between 10^{-13} and 10^{-8}, respectively.

would be used with various biological extracts, 8 U of RNasin was included in our standard protocol.

4. Discussion

We described modifications to the PBRT reaction that retain the high sensitivity of the original assays but eliminate the background signals inherent to those assays. The use of thermostable DNA polymerases that have low RNA-dependent DNA polymerase activity and the inclusion of an RNase incubation prior to the PCR amplification of the cDNA product effectively eliminated the background signal of the assay. The use of the two RNA templates employed in the original assays, BMV RNA (Silver et al., 1993) and MS2 RNA (Pyra et al., 1994), has been extended to include a third commercially-available RNA, that of the RNA genome of tobacco mosaic virus (TMV). There is no a priori reason why additional RNA templates could not be used in the PBRT assay. In fact, the genome of influenza virus has been used successfully (Robertson, personal communication). The modified assay has approximately equivalent sensitivities with three RNA templates and three thermostable DNA polymerases. Using the specific activity of the AMV RT provided by the manufacturer, detection can be as low as 8 molecules of RT. Based on there being between 20 and 100 molecules of RT in a single virion of a retrovirus (Bauer and Temin, 1980; Kacian et al., 1971; Krakower et al., 1977; Layne et al., 1992; Panet et al., 1975; Stromberg et al., 1974), this sensitivity translates into a theoretical detection that is equivalent to between 1 and 10 virions. This value assumes that all virions have functional RT molecules and that all of the RT molecules within a virion are active, neither of which is known. Therefore, the actual level of detection may be lower. Nevertheless, since the number of physical particles to infectious particles varies from $10–10^3$ to 1 for C-type viruses (Smith, 1974; Wilson et al., 1994) to between $10^3–10^4$ to 1 for HIV-1 (Kimpton and Emerman, 1992), the PBRT assay can detect the presence of retroviruses at a level of less than an infectious unit.

Several variables were tested for their contribution to the sensitivity of the PBRT assay. The time of incubation for the RT reaction was found to be important. While Silver et al. (1993) used a 1-h incubation, Pyra et al. (1994) used an incubation of 5 h. In our experience, an incubation of 1 h was insufficient to obtain high sensitivities, and RT reaction times of between 3 and 5 h were routinely used. Incubations for longer than 5 h were not tested.

Increasing the number of PCR cycles could also increase the sensitivity of the reaction. Silver et al. (1993) used 40 cycles, while Pyra et al. (1994) found that 25 cycles were sufficient and more cycles increased the background of their PERT reaction. With the reduced assay backgrounds obtained incorporating the modifications described in this paper, we found that 35 cycles were required to reach the sensitivity of detecting around 10 RT molecules. Using a nested PCR reaction may increase the sensitivity, and this method has been used by Robertson and colleagues (personal communication).

The method of quantification used here was to cut out the bands from the gel and count the radioactivity by liquid scintillation spectrometry. While this method is simple, the PBRT activity could obviously be quantified by other methods, such as phosphorimage analysis.

Whether a separate primer is used for the RT reaction, as in the Silver-Repaske assay, or whether the downstream primer is used for both

the RT reaction and the PCR, as in the PERT assay, was tested using primers designed for use with TMV RNA as template. The sensitivities of the PBRT assays were found to be within the 10-fold range found for the assay to assay variability and therefore it is concluded that there is no advantage of using a third primer.

It was found that the amount of product is proportional to the log of the dilution over a three to four log range. This is perhaps surprising in light of the fact that multiple steps are involved in the PBRT assay and the cycled primer extension reaction quickly reaches a maximum. Importantly, the assay is reproducible within a 10-fold range and does reflect the amount of RT activity in the samples.

There are several potential uses for such sensitive RT assays:

1. They can be used to reveal the presence of low levels of retrovirus contamination in biological products destined for medical or veterinary use.
2. Because RT assays measure an enzymatic activity rather than probe for the viral genome, they do not require that the sequence of the retroviral genome is known and therefore have the capacity to detect novel retroviruses in diseases for which a viral aetiology is suspected but not yet demonstrated.
3. These assays may be used to detect the presence of HIV in individuals with low viral burdens. Certain long term non-progressors have low HIV loads as do some HIV-infected patients receiving anti-viral therapies, particularly the novel triple combination therapies that include protease inhibitors. PBRT assays have the capacity to detect all known as well as unknown HIV-1 and HIV-2 strains without regard to their genotypes. In fact, the PERT assay has been applied successfully to the detection of HIV-1 in the plasma of infected individuals (Böni et al., 1996). In that study, the PERT assay was found to be at least as sensitive as RNA PCR and more reproducible. Also, using the PERT assay, Schüpbach et al. (1996) detected an in vitro transmissible agent with RT activity from a blood donor with a borderline HIV-1 - positive Western analysis. Whether this agent is a divergent HIV strain or another type of virus is not known.
4. Such a highly sensitive assay could also be used to probe for the presence of low levels of RT activity in cellular enzymes such as DNA and RNA polymerases and telomerases. It is possible that an RNA-dependent DNA polymerase activity may be present in several host enzymes, and this may be important to the function of those proteins. While the demonstration that a cellular DNA-dependent DNA polymerase has a low-level RNA-dependent DNA polymerase activity does not necessarily mean that it is used for its normal function, this finding may assist in understanding its mechanism of action and may even suggest evolutionary relationships between polymerases.

Despite the potential power of the PBRT assays, it is their very sensitivity that results in their greatest weakness. They can detect RNA-dependent DNA polymerase activity in DNA polymerases that are not retroviral reverse transcriptases. For example, as shown here, several thermostable DNA polymerases score positive in the assay. Lugert et al. (1996) have shown that mammalian DNA polymerases α and γ have PBRT activity when using BMV RNA as template in a modified Silver-Repaske assay, whereas DNA polymerase β does not. Furthermore, as shown in Fig. 6 and reported by others (Böni et al., 1996; Lugert et al., 1996; Silver et al., 1993), lysates of mammalian cells have PBRT activity. This activity is likely to have multiple sources, since not only do certain DNA polymerases have RT activity (Lugert et al., 1996; unpublished results), but several mammalian cell lines have been found to produce particles that have PBRT activity (Patience et al., 1996). In addition, there are sequences in the mammalian genome that have the potential to produce RT activity. The L1 element has been shown to be able to produce a functional RT when it is expressed in yeast (Dombroski et al., 1994), and several cells produce intracisternal A or R particles, which have also been shown to have RT activity (Rossignol et al., 1975; Salganik et al., 1985).

Therefore, the challenge for the future is not the ability to detect the presence of RT activity, since these PBRT assays are highly sensitive, but rather to develop ways to discriminate between an RNA-dependent DNA polymerase activity of a cellular DNA polymerase and an RT activity associated with a retrovirus. Until assays can be developed that can distinguish reliably between a retroviral RT activity and an RNA-dependent DNA polymerase activity from another source, the usefulness of the PBRT assay to reveal the presence of low levels of retroviruses in biological products or in patient material may be limited.

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References

Bauer, G., Temin, H.M., 1980. Radioimmunological comparison of the DNA polymerases of avian retroviruses. J. Virol. 33, 1046–1057.

Böni, J., Pyra, H., Schüpbach, J., 1996. Sensitive detection and quantification of particle-associated reverse transcriptase in plasma of HIV-1-infected individuals by the product-enhanced reverse transcriptase (PERT) assay. J. Med. Virol. 49, 23–28.

Dombroski, B.A., Feng, Q., Mathias, S.L., Sassaman, D.M., Scott, A.F., Kazazian, H.H., Jr., Boeke, J.D., 1994. An in vivo assay for the reverse transcriptase of human retrotransposon L1 in Saccharomyces cerevisiae. Mol. Cell. Biol. 14, 4485–4492.

Heneine, W., Yamamoto, S., Switzer, W.M., Spira, T.J., Folks, T.M., 1995. Detection of reverse transcriptase by a highly sensitive assay in sera from persons infected with human immunodeficiency virus type 1. J. Infect. Dis. 171, 1210–1216.

Johnson, D.A., Gautsch, J.W., Sportsman, J.R., Elder, J.H., 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Analytical Techniques 1, 3–8.

Jones, M.D., Foulkes, N.S., 1989. Reverse transcription of mRNA by Thermus aquaticus DNA polymerase. Nucleic. Acids Res. 17, 8387–8388.

Kacian, D.L., Watson, K.F., Burny, A., Spiegelman, S., 1971. Purification of the DNA polymerase of avian myeloblastosis virus. Biochim. Biophys Acta 246, 365–383.

Kimpton, J., Emerman, M., 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. J. Virol. 66, 2232–2239.

Krakower, J.M., Barbacid, M., Aaronson, S.A., 1977. Radioimmunoassay for mammalian type C viral reverse transcriptase. J. Virol. 22, 331–339.

Layne, S.P., Merges, M.J., M., D., Spouge, J.L., Conley, S.R., Moore, J.P., Raina, J.L., Renz, H., Gelderbloom, H.R., Nara, P.L., 1992. Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus. Virology 189, 695–714.

Lugert, R., König, H., Kurth, R., Tönjes, R.R., 1996. Specific suppression of false-positive signals in the product-enhanced reverse transcriptase assay. BioTechniques 20, 210–215.

Panet, A., Baltimore, D., Hanafusa, T., 1975. Quantitation of avian RNA tumor virus reverse transcriptase by radioimmunoassay. J. Virol. 16, 146–152.

Patience, C., Simpson, G.R., Colletta, A.A., Welch, H.M., Weiss, R.A., Boyd, M.T., 1996. Human endogenous retrovirus expression and reverse transcriptase activity in the T47D mammary carcinoma cell line. J. Virol. 70, 2654–2657.

Peden, K., Emerman, M., Montagnier, L., 1991. Changes in growth properties on passage in tissue culture of viruses derived from infectious molecular clones of HIV-1_{LAI}, HIV-1_{MAL}, and HIV-1_{ELI}. Virology 185, 661–672.

Pyra, H., Böni, J., Schüpbach, J., 1994. Ultrasensitive retrovirus detection by a reverse transcriptase assay based on product enhancement. Proc. Natl. Acad. Sci. USA 91(4), 1544–1548.

Reed, K.C., Mann, D.A., 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. 13, 7207–7221.

Rossignol, J.M., Kress, M., de Recondo, A.M., 1975. Reverse transciptase activity associated with R-type virus-like particles of SV40-transformed hamster cells (TSV5 clone 2). Intervirology 5, 273–292.

Salganik, R.I., Tomsons, V.P., Pyrinova, G.B., Korokhov, N.P., Kiseleva, E.V., Khristolyubova, N.B., 1985. Reverse

transcriptase of rat liver associated with the endogenous retrovirus related to the mouse intracisternal A-particles. Biochem. Biophys. Res. Commun. 131, 492–499.

Schüpbach, J., Pyra, H., Jendis, J., Tomasik, Z., Böni, J., 1996. Isolation of an in vitro transmissible agent with reverse transcriptase activity from a blood donor with a borderline-positive HIV-1 serology for more than five years. Clinical and Diagnostic Virology 5, 197–203.

Silver, J., Maudru, T., Fujita, K., Repaske, R., 1993. An RT-PCR assay for the enzyme activity of reverse transcriptase capable of detecting single virions. Nucleic. Acids Res. 21, 3593–3594.

Smith, R.E., 1974. High specific infectivity avian RNA tumor viruses. Virology 60, 543–547.

Stromberg, K., Hurley, N.E., Davis, N.L., Rueckert, R.R., Fleissner, E., 1974. Structural studies of avian myeloblastosis virus: comparison of polypeptides in virion and core component by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Virol. 13, 513–528.

Wilson, C.A., Eiden, M.V., Marsh, J.W., 1994. Quantitative micro p30 and reverse transcriptase assays for Moloney murine leukemia virus. J. Virol. Methods 48, 109–117.

Yamamoto, S., Folks, T.M., Heneine, W., 1996. Highly sensitive qualitative and quantitative detection of reverse transcriptase activity: optimization, validation, and comparative analysis with other detection systems. Journal of Virological Methods 61, 135–143.

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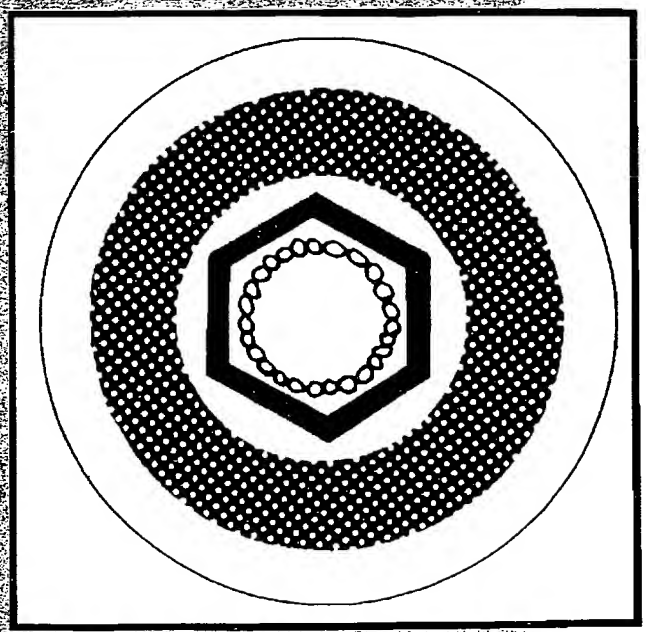

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Effects of primer–template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies

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ABSTRACT

We investigated the effects of various primer-template mismatches on DNA amplification of an HIV-1 *gag* region by the polymerase chain reaction (PCR). Single internal mismatches had no significant effect on PCR product yield while those at the 3'-terminal base had varied effects. A:G, G:A, and C:C mismatches reduced overall PCR product yield about 100-fold, A:A mismatches about 20-fold. All other 3'-terminal mismatches were efficiently amplified, although the G:G mismatches appeared to be more sensitive to sequence context and dNTP concentrations than other mismatches. It should be noted that mismatches of T with either G, C, or T had a minimal effect on PCR product yield. Double mismatches within the last four bases of a primer-template duplex where one of the mismatches is at the 3' terminal nucleotide, in general, reduced PCR product yield dramatically. The presence of a mismatched T at the 3'-terminus, however, allowed significant amplification even when coupled with an adjacent mismatch. Furthermore, even two mismatched Ts at the 3'-terminus allowed efficient amplification.

INTRODUCTION

Viral genomes, particularly those of RNA viruses and retroviruses, contain multiple base alterations, additions, duplications, and deletions. The variability of these viruses has been attributed to the low fidelity and lack of proofreading functions of the polymerases responsible for their replication (1). In addition, the fidelity of RNA polymerase II which plays a critical role in the retroviral life cycle, and of the reverse transcriptase must be taken into consideration. The repeated rounds of replication required for infection further magnify variability. The role that these viral variants play in the natural history of infection is only beginning to be ascertained and appears to vary with each class of virus.

The polymerase chain reaction (PCR)(2–4) has proven to be a sensitive and specific assay for the detection of retroviral sequences (see for example 5–8). Because of the inherent genetic variability of these viruses, detection by PCR requires the identification of primers that will recognize the viral variants. To ensure the efficient amplification and detection of such viruses, we selected primers that amplify regions of viral genomes that contain either conserved amino acid or nucleic acid sequences. In the former case, the selection of conserved regions encoded by amino acids with minimal codon degeneracy reduces the number of possible oligonucleotides required to prime the region (9). In the latter case, the identification of regions conserved in a large number of sequenced isolates will aid in the selection of primer pairs that will amplify most viral variants (Human Retroviruses and AIDS, 1989, Los Alamos National Laboratory).

The ability for an oligonucleotide to serve as a primer for PCR is dependent on several factors, including a) the kinetics of association and dissociation of primer-template duplexes at the annealing and extension temperatures, b) the effects on duplex stability of mismatched bases and their location, and c) the efficiency with which the polymerase can recognize and extend a mismatched duplex. Since single mismatches at or near the terminal 3' base of a primer are known to affect both oligonucleotide stability and efficiency of polymerase extension, they should effect PCR more dramatically than mismatches at other positions (10).

Several investigators have begun to evaluate the effect of single 3'-terminal mismatches (11–15). In this study, we evaluated the effect on PCR of various primer-template mismatches used for amplification of a region of the human immunodeficiency virus type 1 (HIV-1). The importance of HIV detection (5,6,8 16–22) coupled with the heterogeneity of the HIV genomes (23–26), suggested that primers for this virus would serve as an important model for this study.

MATERIALS AND METHODS

HIV Model System

A 130 bp region of *gag* in HIV-1 (NT 1377–1506 of HIVSF2) was amplified by pairing the upstream primer, SK145 or its derivatives (Table I) with the downstream primer SK150 (5' TGCTATGTCACTTCCCCTTGGTTCTCTC). Oligonucleotide SK102 (5' GAGACCATCAATGAGGAAGCTGCAGAAT-GGGAT) hybridizes to a region within the amplified product and is used as the probe in Southern blot and oligomer hybridization analyses (see below). The oligonucleotides described here also amplify HIV-2.

The templates used in this study were either a recombinant plasmid that harbors an HIV-1 genome, Z6 (gift of C.Y. Ou and G. Schochetman, CDC), or were templates generated by PCR (see below). The sequence of plasmid Z6 at the primer annealing sites was determined by amplification and cloning into M13 of a 300 bp fragment that spans the 130 bp fragment of interest. Sequence analysis shows that SK145 is homologous to Z6 and SK150 differs from plasmid Z6 at a single nucleotide 22 bases from the 3′ end. The single base alteration in SK150 is to more efficiently provide amplification of HIV-2. The sequence of SK150 therefore is a hybrid sequence varying by a single base from the type 1 and type 2 viruses.

Table I. Sequence of SK145 and its Derivatives.

PRIMER DESIGNATION	PRIMER SEQUENCE	POSITION OF BASE ALTERATION
SK145	AGTGGGGGGACATCAAGCAGCCATGCAAAT	NONE
289	----------A	3′
290	----------G	3′
291	----------C	3′
292	----------T-	-1
293	----------G-	-1
294	----------C-	-1
295	----------T--	-2
296	----------G--	-2
297	----------C--	-2
298	----------T---	-3
299	----------G---	-3
300	----------C---	-3

Dashes represent nucleotides that are identical to SK145. Upper case letters denote mismatches relative to SK145.

Oligonucleotides

Two sets of oligonucleotides were synthesized and used for this study. One set of oligonucleotides that differed from SK145 at one of the last 4 bases of the 3′-terminus was used to study the effects of mismatches on amplification (Table I). A comprehensive study of the effects of various primer:template mismatches requires not only primers with base alterations but also templates with various base alterations. We chose to generate templates with the desired change(s) by PCR amplification of plasmid Z6 with a set of 'mutagenic' oligonucleotides. The 'mutagenic' oligonucleotides (SK277 to SK288 and SK312 to SK320) are identical to SK145 at their 5′ ends but have incorporated into their sequence the altered base(s) and are

Table II. Sequence of "Mutagenic" Primers

PRIMER DESIGNATION	PRIMER SEQUENCE	POSITION OF BASE ALTERATION(S)
SK145	AGTGGGGGGACATCAAGCAGCCATGCAAAT	NONE
277	----------Agtta	3′
278	----------Ggtta	3′
279	----------Cgtta	3′
280	----------Ttgtt	-1
281	----------Gtgtt	-1
282	----------Ctgtt	-1
283	----------Tatgt	-2
284	----------Gatgt	-2
285	----------Catgt	-2
286	----------Taatg	-3
287	----------Gaatg	-3
288	----------Caatg	-3
312	----------TCgtta	3′,-1
313	----------CCgtta	3′,-1
314	----------GCgtta	3′,-1
315	----------TAgtta	3′,-1
316	----------CAgtta	3′,-1
317	----------GAgtta	3′,-1
318	----------TGgtta	3′,-1
319	----------CGgtta	3′,-1
320	----------GGgtta	3′,-1

Dashes represent nucleotides that are identical to SK145. Upper case letters denote mismatches relative to SK145. Lower case letters denote bases extended beyond mismatch.

extended by 4 bases beyond the 'mutagenic' base(s) (Table II). We assumed that the 4 additional complementary bases at the 3' end would facilitate extension by *Thermus acquaticus* (*Taq*) DNA polymerase. The predicted Tms for SK145 and SK150 are 83°C and 74°C, respectively, in 0.1M NaCl at a concentration of $1X10^{-6}$M using an algorithm proposed by Breslauer (27,28). The internal G:A mismatch between SK150 and plasmid Z6 should have little effect, if any, on the thermostability of the duplex (29).

The oligonucleotides were synthesized on a Model 8750 DNA synthesizer (Milligen/Biosearch, San Rafael, CA) using long-chain alkylamine controlled pore glass supports and beta-cyanoethyl N,N-diisopropyl phosphoramidites (American Bionetics, Hayward, CA). Standard ancillary reagents and synthesis protocols were used (30,31). The oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis which removes contaminating truncated sequences and subsequently desalted by reversed-phase HPLC. Since the oligonucleotides were synthesized 3' to 5', 3' truncated oligonucleotides were not present in the preparations. Base composition analysis was performed to assure that the isolated fragment contained the appropriate number and ratio of nucleosides.

DNA Amplifications

DNAs were amplified in 100 μl reaction volumes with 50 pmoles of each primer, 2 units AmpliTaq DNA polymerase (Perkin-Elmer Cetus Inc.) and either 800, 50, or 6 μM total dNTPs in a buffer containing 10 mM Tris pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$. The concentration of primers used was determined by optical density and evaluated by gel electrophoresis.

All amplifications were performed using either plasmid Z6 or PCR-generated products as templates. To generate the base-altered templates, ten thousand copies of Z6 plasmid were amplified in the absence of human genomic DNA. The amount of base altered templates generated by PCR was estimated by comparing the intensities of the ethidium bromide stained product bands to known amounts of marker DNA. Each template was diluted and normalized to the desired concentration before use. Since each PCR-generated product was diluted by at least 10^6 for use as template, contribution of original Z6 molecules to subsequent amplifications is highly unlikely. To determine the effects on PCR of primer-template mismatches, concentrations of approximately 10,000 and 100 copies of either plasmid Z6 or PCR-generated templates were amplified in the presence of 1 μg human placental DNA. Plasmid DNA concentration was determined by optical density at 260nm.

Samples were amplified by 30 repeated cycles on a DNA Thermal Cycler (Perkin-Elmer Cetus, Inc.) using the following parameters: DNA denaturation, 25 sec at 95°C; primer annealing, 25 sec at 55°C; and primer extension, 1 min. at 72°C. For generation of templates with two consecutive mismatches at the 3' end, 'mutagenic' oligonucleotides with 2 consecutive mismatches with plasmid Z6 at positions 5 and 6 from the 3' terminus (SK312 to SK320) were used. In order to achieve more efficient amplifications, a lowering of the annealing temperature to 37°C was necessary. Presumably, these two consecutive mismatches were sufficient to disrupt the stability of the AT-rich 3' end. All amplifications were performed in triplicate and repeated separately with multiple stock solutions.

Detection

The analysis of two different target concentrations and the dramatic differences in the overall yield of the amplification reactions required the use of different detection schemes. Products of high copy target amplifications were analyzed by NuSieve agarose gel electrophoresis and ethidium bromide staining, and confirmed by Southern blot analysis with a 32P end-labeled SK102 probe (specific activity of 1.5−3μCi/pmole) (32). Oligomer hybridization was used to analyze the products of low copy target amplifications (18). As described here, the PCR product yield from any one reaction varies by no more than 50% from sample to sample and run to run. For simplicity, this variability was not incorporated into Table III.

Table III. Relative amplification efficiencies of 3'-terminal mismatches in the presence of 800 μM dNTPs. Product yields were normalized to the perfect matches (1.0).

		Primer 3' Base			
		T	C	G	A
Template 3' Base	T	1.0	1.0	1.0	1.0
	C	1.0	≤.01	1.0	1.0
	G	1.0	1.0	1.0	≤.01
	A	1.0	1.0	≤.01	0.05

RESULTS AND DISCUSSION

Mismatches at the 3' terminus of a primer

The synthesis of oligonucleotides that differ only at the 3'-terminal base, coupled with templates that contain different bases at the corresponding position, provide a system to test the effect of mismatches on PCR. Although amplification of a subset of these templates and primers would provide information on all mismatches, we chose to generate and amplify each template with the four available primers for two reasons. First, we wanted to determine whether the effects of mismatches on PCR were symmetrical. For example, would a G:T (primer:template) mismatch have the same effect on PCR as a T:G mismatch? Second, an intrinsic 3' to 5' exonuclease activity has not been demonstrated for *Taq* polymerase (33,34), therefore, an asymmetric effect of mismatches on PCR may reflect 'context' effects (role of flanking sequences) on the efficiency of extension of a mispaired primer-template.

The results from the 3' mismatch experiments are summarized in Table III. Under the conditions used, and with 800 μM total dNTPs, most mismatches did not significantly affect amplification. In fact, the presence of a T at the 3' end of the primer provided efficient amplification irrespective of the corresponding nucleotide in the template. The mean amplification efficiency per cycle of SK145−150 and these mismatched primer pairs is approximately 85% after 30 cycles. Examination of PCR product after 25 cycles suggested that the efficiency of amplification between 25 and 30 cycles was similar to the amplification efficiency of the earlier cycles. The amplification efficiencies were determined as previously described (3). An A:A (primer:template) mismatch resulted in a 20-fold reduction in overall product yield and A:G, G:A and C:C resulted in approximately a 100-fold reduction. The effects of the mismatches on PCR were symmetrical. For example, both A:G and G:A mismatches were equally detrimental to PCR under these conditions. A representative gel and Southern blot analysis of these amplifications is shown in Figure 1 (brackets 1−3).

Variations in the reaction components and annealing temperatures may affect amplification by mismatched primers. In this study, we examined the effect on amplification of lowering the dNTPs to 50 μM and 6 μM. The results from the

Figure 1. Representative amplifications of plasmid Z6 DNA with primers that were mismatched with template. Samples were amplified in triplicate and visualized on NuSieve agarose gel (Panel A) and analyzed by Southern blot (Panel B). Plasmid Z6 was amplified by coupling SK150 with a primer that had either an A:A, G:A or C:A primer:template mismatch at the terminal 3' position in 1, 2, and 3, respectively; T:T, G:T or C:T mismatches one nucleotide from the 3' terminus in 4, 5 and 6, respectively. The positive control (+) represents amplification of plasmid Z6 DNA with a perfectly matched primer. The arrow indicates the position of the product of interest.

amplifications with 50 μM dNTPs were similar to those with 800 μM dNTPs with the exception that the G:G mismatch amplified poorly. In 6 μM dNTPs, only perfectly matched 3'ends were extended with the exception of a T:G mismatch. The overall product yield with this level of dNTPs was at least 10 to 20-fold less than with 800 μM. However, the absence of detectable product may reflect small differences in amplification efficiency rather than complete inhibition of extension. The lower amplification efficiency is more likely a result of lower dNTP concentrations than the higher relative concentration of $MgCl_2$, as this primer pair amplifies efficiently with a broad range of $MgCl_2$.

One of the important factors that affect PCR product yield is the relative efficiency with which the polymerase extends from a mismatched primer-template duplex. Once extension from a mismatched primer occurs, the resultant product and the complement synthesized in subsequent cycles are fully matched with both primers. The molecules with termini defined by both primers accumulate exponentially. On the other hand, products of extensions from the original plasmid template have only one defined terminus and accumulate linearly. Therefore, the contribution of mismatched extension products to total yield, although significant in early cycles, becomes negligible in later cycles. The observed reductions in PCR product reflect dramatic decreases in the efficiency of mismatch extension relative to perfect match extension. If one assumes that mismatch extensions only contribute significantly in the first ten cycles (at which point the mismatch extended templates represent at best 2% of the fully-matched templates) and that the mean efficiency per cycle after cycle ten is 85%, then our studies using 800 μM dNTPs, suggest that an A:A mismatch has an average per cycle efficiency of 50% and the A:G, G:A and C:C mismatches at only an average per cycle efficiency of 16% relative to perfect matches during the first ten cycles. Changes in the reaction conditions such as the concentration of magnesium chloride and annealing temperatures are expected to alter the results described here.

Although the product yields obtained by amplification of a common template with different primers can be directly compared, the products generated from amplification of different templates with common primers may vary somewhat as a result of differences in the amount of PCR-generated template used to seed each reaction. Although the templates were diluted and normalized, the possibility exists that minor differences in product yield merely reflect differences in initial template copy number. Minor differences in template and primer concentrations, however, would not account for the dramatic effects on PCR that

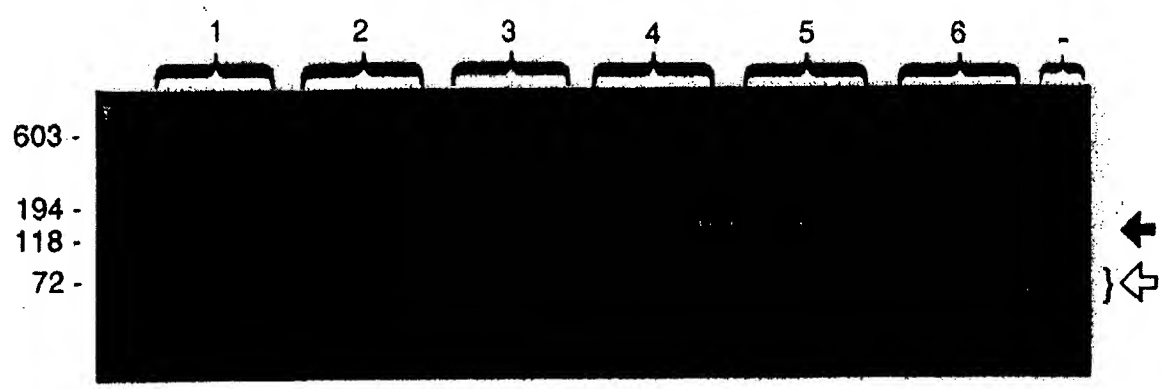

Figure 2. Representative amplifications of plasmid Z6 DNA with primers that differ by a single nucleotide. The plasmid DNA was amplified in triplicate in the presence of 1 ug human placental DNA by coupling SK150 with SK295–300 in (1–6), respectively. Amplification of human placental DNA in the absence of plasmid Z6 is shown in the lane designated (–). The solid arrow shows the position of the desired product; the open arrow shows the positions of the primer-dimers.

were observed with some mismatches. All amplifications were first performed on a high level of target molecules and were repeated on a low level of target. It is expected that effects on PCR yield would be observed in the presence of few targets that may otherwise be masked when amplifying high levels of target molecules. The results from the low target experiments were similar to those obtained from the high target experiments.

Single internal mismatches

In the process of generating templates with altered bases, we demonstrated that a single mismatch 3 residues from the 3′ terminal base of a primer can be efficiently extended without modification of amplification reaction conditions. Similarly, mismatches 1, 2, or 3 bases from the 3′ nucleotide of primers had no apparent affect on overall PCR product yield (for representative amplifications, see figure 1, brackets 4–6). Of particular note are the differences in non-specific amplification products of various molecular weights generated by these reactions. Amplification of the various 'mutated' templates with a common primer pair (SK145–150) resulted in background products that were similar in size. In contrast, amplification of a common template with primers that differ by a single base gave rise to background amplification products that varied dramatically in intensity and size (Figure 2). These data suggest that minor modifications in the primer sequence can dramatically affect the specificity of the amplifications.

In addition, a major product that migrates at approximately the distance expected of the sum of the two primers is often observed in amplification reactions. These products have been cloned and analyzed from several different primer-pair systems (B. Watson, S. Kwok, personal communications). Sequence analysis reveals a fragment bearing the sequence of one of the primers contiguous with the sequence of the complement of the other primer and hence, the product has been termed 'primer-dimer'. Primer-dimers containing a sequence homologous to one of the primers contiguous with its complement have not been identified. It is thought that such a molecule would form a stable hairpin loop structure and would therefore not readily amplify. In some cases, the two primer sequences were separated by 1–10 nucleotides. The mechanism by which these primer:dimers form is not clear. Two alternative explanations can be used to explain these amplification products. First, one of the single stranded primers binds to the polymerase and uses the 3′ end of the second primer as a template for extension. Second, genomic DNA and trace quantities of nucleic acids present in the reactions may contain sequences that are contiguous and similar enough to the primers that they serve as a template to generate these molecules. Given that the downstream primers in these reactions are identical and that the upstream primers differ by only a single base change, it is interesting that amplification of a common template gave rise to not only background products of different sizes and intensities but also primer-dimers of different sizes and intensities (Figure 2).

Multiple mismatches

The effect on PCR of mismatches in a primer:template duplex depends largely on the position and nature of the mismatches. We demonstrated above that some base mismatches (C:T, G:T, T:T, G:G, T:G, T:C, A:C, C:A,) at the 3′-terminus did not have a significant effect on PCR using the conditions described. Although some 3′ mismatches affected amplification, single primer-template mismatches 1 (i.e. penultimate base), 2 or 3 bases from the 3′-nucleotide of a primer did not have a significant affect on PCR product yield. However, when mismatches not involving a T at the 3′ end were coupled with any additional mismatch either 1, 2, or 3 bases from the 3′-nucleotide, PCR product yield was reduced by at least 100-fold (data not shown). However, a T mismatch at the 3′ terminus coupled with an additional mismatch at the penultimate position, resulted in only a 5–10 fold reduction in product yield. Furthermore, the presence of 2 Ts at the 3′ terminus of the primer resulted in overall product yields that were reduced by only 2–5 fold when compared to amplifications by the perfectly matched primers. For these studies, primer pairs SK289–150, SK290–150, SK291–150 or SK292–150 were used to amplify templates generated by amplification of plasmid Z6 with primer pairs SK280–150 through SK288–150 or SK312–150 through SK320–150 (see Tables I and II).

In the generation of templates with two consecutive base alterations, we found that mismatches 5 and 6 residues from the 3′ end had a detrimental effect on amplification when a 55°C annealing temperature was used. However, by lowering the annealing temperature to 37°C, a more efficient amplification was achieved. Because of the AT richness of the 3′ terminus of

these primers, we speculate that the presence of 2 consecutive mismatches 5 and 6 bases from the 3′ terminus sufficiently destabilized the 3′ terminus such that extension by *Taq* DNA polymerase was very inefficient.

The presence of multiple mismatches at least 8 bases from the 3′ terminus of SK145 does not appear to have a significant effect on PCR under the conditions used. A molecular clone of HIVMAL (35) has five mismatches with SK145 and a single G:A mismatch 21 bases from the 3′-terminus with SK150.

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SK145   AGTGGGGGGACATCAAGCAGCCATGCAAAT
HIVMAL  ---T--A-----C--G-----T--------
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One would predict that the Tm of SK145 on the HIVMAL template might be at least 10°C lower. Despite these mismatches, HIVMAL was efficiently amplified under the conditions used. However, when the annealing temperature was raised to 60°C, PCR product yield was significantly reduced, and at 65°C, amplification was not detected. These results underscore the importance of using less stringent annealing temperatures for the amplification and detection of highly variable targets.

CONCLUSIONS

Several conclusions can be drawn from this study. Certain mismatches at the terminal 3′ position (ie. T:C, T:G, T:T, G:G, and A:C) appear to amplify as efficiently in PCR as the fully complementary primer-template duplex, under these conditions, while an A:A mismatch moderately reduced PCR amplification efficiency per cycle, and A:G and C:C mismatches dramatically reduced PCR amplification efficiency. Although G:G mismatch amplified efficiently at 800 μM dNTPs, the product yield in 50 μM dNTPs was dramatically reduced. Whereas some 3′-terminal mismatches were poorly extended, single base mismatches between the primer and template either one, two or three bases from the 3′ nucleotide of the primer can be extended without a significant effect on overall product yield. However, when coupled with an additional mismatch within the last four bases, overall PCR product yield from a 3′ terminal mismatched primer is drastically reduced. In contrast, oligonucleotides with a T mismatch at the 3′ terminus when coupled with an additional mismatch at the penultimate position served efficiently as primer for amplification. Further, the presence of 2 Ts at the 3′ terminus enabled amplification of templates with mismatches at both positions irrespective of the nucleotides involved.

A priori, the thermodynamic stability of base mismatches cannot explain the results observed here. The efficiency at which polymerase extends from a mismatched base pair depends on a number of complex interactions. First, the overall stability of the primer-template may determine the likelihood that polymerase binds to a duplex. Second, extension by polymerase probably reflects recognition of the base stacking, hydrogen bonding, and overall steric structure of the terminus to be extended. These factors may themselves interact since hydrogen bonds may facilitate stacking by bringing the bases in proximity and vice versa. Although numerous studies have begun to describe the calculated and measured stabilities of the various mismatches alone and relative to sequence context (10,36), the simplest interpretation of our data is that purine-purine mismatches do not extend efficiently while pyrimidine-pyrimidine and purine-pyrimidine mismatches do extend efficiently under these conditions. The G:G and C:C mismatches serve as exceptions to these conclusions. Perhaps the stacking forces of the G residue with the penultimate base and the hydrogen bonded structure proposed by Abou-ela, et al (29) play a role in efficient extension of this mismatch by polymerase. On the other hand, the proposed sugar-phosphate constrained structure with only one hydrogen bond for a C:C mismatch may disrupt the 3′-terminal structure to the extent that efficient extension is not possible (29). Extension of the primer-template duplex from a G:T mismatch perhaps would have been expected since it contributes neither a stabilizing nor destabilizing influence to the nucleic acid duplex (37). The efficient extension of a T:C or T:T mismatch was not, however, expected. Recent studies by M.Goodman and colleagues on the relative extension efficiencies of mismatched termini by AMV reverse transcriptase and DNA polymerase alpha are in general agreement with our results (38). The extent to which one can extrapolate our data to other primer pair-template systems will require additional studies. The extent to which the three contiguous A's near the three terminus contributes to these results is unclear. However, our preliminary experiments with other HIV and HTLV primer pairs support the general applicability of these observations. Included in these experiments are data suggesting that the efficiency of extension of a G:G mismatch may be dependent on sequence context as well as on the concentration of deoxynucleoside triphosphates.

Several investigators have begun to explore the effects of mismatches on the amplification of cellular sequences. A direct comparison of our results with those previously published is difficult due to differences in reaction conditions (buffer, primer concentration, annealing temperature), target copy number, primer length and sequence context. In some studies, the primers were short (12 – 16 bases) and therefore a 3′ mismatch may have more dramatically affected overall primer:template duplex stability as well as the ability for the polymerase to catalyze extension.

Our results are in agreement with those of Ehlen et al. (13) where the amplifications were carried out under similar reaction conditions and with primers of similar length. Ehlen et al. (13) demonstrated that at 200 μM dNTPs, a C:T mismatch can be extended where as at 2 μM dNTPs C:T, A:C and C:C mismatches were not extended. Using similar conditions but with primers that were 16 bases long, Nichols et al. (12) also showed that an A:A mismatch was not extended. Wu et al. (11), again under similar conditions but with primers that were 14 bases in length, presented evidence that A:A and T:T mismatches were not extended at an annealing temperature of 55°C but were extended at annealing temperatures of 44°C and 50°C. The extension of the A:A mismatch at the lower annealing temperature is contrary to our finding. The studies of Okayama et al. (15) were also carried out under similar conditions but with shorter primers. Their results are contrary to all other studies in that 10 of the 12 possible mismatches tested prevented significant amplification; the A:G and C:T mismatched were not evaluated.

Newton et al. (14) demonstrated the use of 3′-terminal mismatched primers for allele-specific amplifications. In their study, G:T, T:G, A:C, and C:A, mismatches were extended by *Taq* DNA polymerase whereas A:A, T:T, and C:T mismatches were refractory to extension. The C:T and T:T results are contrary to our findings and may reflect the substantial differences in the reaction conditions. Consistent with Newton et al., we found that single internal mismatches did not significantly affect

PCR product yield. Furthermore, the placement of an additional internal mismatch to extendable 3'-terminal mismatches proved refractory to amplification.

Implications of this study extend to multiple areas. First, primers for nucleic acid templates from pathogens known to vary in sequence would benefit from a single T or perhaps double T at the extreme 3' end so as not to obviate amplification because of mismatched bases at 3' end. The HIV-1 model system used in this report is of particular importance given the extensive heterogeneity within and among infected individuals. Ideally, the primers used in a diagnostic assay for this virus should not only have the desired sensitivity and specificity on contemporary isolates but should also be engineered to accommodate a degree of divergence expected in future isolates. The observations noted in this study may contribute to the design of primers for the detection of other variable pathogenic viruses such as the human papilloma viruses and RNA viruses. Second, this information will aid in the design of primers used in the search for additional members of known virus groups (7,9). For example, the design of primers with T rather than A or G at the 3' terminus may increase the likelihood of extension.

On the other hand, there are applications in which primers need to be designed for allele-specific amplifications. The presence of a 3' terminal A:G or C:C mismatch, and to a lesser extent an A:A mismatch, should bias the amplification to the desired targets. In addition, alterations in the concentration of reaction components (13) and annealing temperature should also effect extension from mismatched primers and therefore should also be exploited.

Finally, we expect that studies similar to those described here will begin to address the specific protein-nucleic acid interactions of polymerases and their substrates so that we may better understand substrate binding and catalysis by this family of enzymes.

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REFERENCES

1. Steinhauer, D.A. and Holland, J.J.(1986) Rapid evolution of RNA viruses. Annu Rev Microbiol **41**,409–433 .
2. Mullis, K.B. and Faloona, F.A. (1987) In Wu R (ed), Methods in Enzymology Vol **155**,335–350.
3. Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T. and Erlich, H.A. (1985) Science **230**,1350–1354.
4. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R. and Horn, G.T. (1988) Science **239**,487–491.
5. Kwok, S., Mack, D.H., Mullis, K.B., Poiesz, B., Ehrlich, G., Blair, D., Friedman-Kien, A. and Sninsky, J.J. (1987) J. Virol. **61**,1690–1684.
6. Ou, C-Y, Kwok, S., Mitchell, S.W., Mack, D.H., Sninsky, J.J., Krebs, J.W., Feorino, P., Warfield, D. and Schochetman, G. (1988) Science **239**,295–297.
7. Kwok, S., Ehrlich, G., Poiesz, B., Kalish, R, and Sninsky, J.J. (1988) Blood **72**,1117–1123.
8. Kwok, S., Mack, D.H., Sninsky, J.J., Erlich, G.D., Poiesz, B.J., Dock, N.L., Alter, H. J., Mildvas, D. and Grieco, M.H. 1989 in Luciw PA, Steimir KS (eds). HIV Detection by Genetic Engineering Methods. pp. 243–255.
9. Mack, D.H. and Sninsky, J.J. (1988) Proc Natl Acad. Sci USA **85**,6977–6981.
10. Petruska, J., Goodman, M.F., Boosalis, M.S., Sowers, L.C., Cheong, C. and Tinoco I. (1988) Proc. Natl Acad Sci USA **85**,6252–6256.
11. Wu, D. Y., Ugozzoli L, Pol B. K. and Wallace R. B. (1989) Proc. Natl Acad Sci USA **86**, 2759–2760.
12. Nichols W. C., Liepnieks J. J., McKusick V. A. and Benson M. D. (1989) Genomies **5**, 535–540.
13. Ehlen T., and Dubeau L. (1989) Biochem. Biophys. Res. Commun. **160**, 441–447.
14. Newton C.R., Graham A., Heptinstall L.E., Powell S.J., Summers C., Kalsheker N., Smith J.C., and Markham A.F. (1989) Nucleic Acids Res. **17**:2503–2516.
15. Okayama H., Curiel D. T., Brantly M. L., Holmes M. D. and Crystal R. G. (1989) J. Lab Clin Med **114**, 105–113.
16. Laure, F., Courgnaud, V., Rouzioux, C., Blanche, S., Verber, F., Burgard, M., Jacomet, C., Griscelli, C. and Brechot, C. (1988) Lancet **2**,538–541.
17. Loche, M. and Mach, B. (1988) Lancet **2**,418–421.
18. Jackson, J.B., Kwok, S.Y., Hopsicker, J.S., Sannerud, K.J., Sninsky, J.J., Edson, J.R. and Balfour H.H. (1989) Transfusion **29**,265–267.
19. Farzadegan, H., Polis, M.A., Wolinsky, S.M., Rinaldo, C.R., Sninsky, J.J., Kwok, S., Griffith, R.L., Kaslow, R.A., Phair, J.P., Polk, F.P. and Saah, A.J. (1988) Ann Intern Med. **108**,785–790.
20. Imagawa, D.T, Lee, M.M., Wolinsky, S.M., Sana, K., Morales, F., Kwok, S., Sninsky, J.J., Nishanian, P.G., Giorgi, J., Fahey, J.L., Dudley, J., Visscher, B.R. and Detels R. (1989) N Eng J Med. **320**,1458–1462.
21. Rogers, M.F., OuC-Y, Rayfield, M., Thomas, P.A., Schoenbaum, E.E., Abrams, E., Krasinski, K., Selwyn, P.A., Moore, J., Kaul, A., Grimm, K.T., Bamji, M., Schochetman, G., and the New York City Collaborative Study of Maternal HIV Transmission and Montefiore Medical Center HIV Perinated Transmission Study Group. (1989) N. Engl. J. Med. **320**,1649–1654.
22. Chadwick, E.G., Yogev, R., Kwok, S., Sninsky, J.J., Kellogg, D.E. and Wolinsky, S.M. (1989) J. Infect. Dis. (in press).
23. Shaw, G.M., Hahn, B.H., Arya, S.K., Groopman, J.E., Gallo, R.C. and Wong-Staal F. (1984) Science **226**,1165–1171.
24. Saag, M.S., Hahn, B.H., Gibbons, J., Li, Y., Parks, E.S., Parks, W.P. and Shaw, G.M. (1988) Nature **334**,440–444.
25. Goodenow, M., Huet, T., Saurin, W., Kwok, S., Sninsky, J. and Wain-Hobson, S. (1989) JAIDS **2**:344–352
26. Meyerhans, A., Cheynier, R., Alberts, S., Seth, M., Kwok, S., Sninsky, J.J., Morfeldt-Manson, L., Asjo, B. and Wain-Hobson, S. (1989) Cell **58**, 901–910.
27. Breslauer, K.J., Frank, R., Blocker, H. and Marky, L.A. (1986) Proc Natl Acad Sci USA **83**,3746–3750.
28. Marky, L.A. and Breslauer, K.J. (1987) Biopolymers **26**,1601–1620.
29. Aboul-ela F., Koh D., Tinoco I. Martin F.H. (1985) Nucleic Acids Res. **13**:4811–4824.
30. Beaucage, S.L. and Caruthers, M.H. (1981) Tetrahedron Letters **22**,1859–1862.
31. Sinha, N.D., Biernat, J., and Koster H. (1984) Nucleic Acids Res. (1984) **12**,4539–4557.
32. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
33. Tindall, K.R. and Kunkel, T.A. (1988) Biochemistry **27**,6008–6013.
34. Lawyer, F.D., Stoffel, S., Saiki, R., Myambo, K., Drummond, R. and Gelfand, D.H. (1989) J. Biol. Chem. **264**,6427–6437.
35. Alizon, M., Wain-Hobson, S., Montagnier, L. and Sonigo, P. (1986) Cell **46**,63–74.
36. Ikuta, S., Takagi, K., Wallace, R.B. and Itakura K. (1987) Nucleic Acids Res. **15**,797–811.
37. Patel D.J., Kozlowski S.A., Ikuta S., Itakura, K. (1984) Biochemistry **23**:3207.
38. Mendelman, L. V., Petruska, J. and Goodman, M. F. J. Biol. Chem. (in press)

Chapter 12

DISTINCTION BETWEEN ALMOST-IDENTICAL DNA SEQUENCES BY POLYMERASE CHAIN REACTION

Jean-Paul Charlieu

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I. INTRODUCTION

Two DNA fragments sometime present very strong sequence homology. For example, the alleles of a gene can differ at only one position, when a point mutation occurs. Several techniques, such as denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), and hybridization with allele-specific oligonucleotides (ASO) allow discrimination between almost-identical DNA sequences.

It has been shown recently[1] that PCR can also be used to distinguish DNA variants in the HIV genome. Based on this study, PCR conditions were developed to distinguish the alpha-satellite subfamilies of chromosomes 13 and 21.[2] Alpha-satellite is a family of tandemly repeated DNA sequences present at the centromeric region of all human chromosomes.[3] Slight variations in the nucleotide sequence of the ~171-bp basic motif and in the distribution of restriction sites define higher order repeats that are specific for one or a few chromosomes.[4] The subfamilies from chromosomes 13 and 21 are almost identical, however, as they share 99.7% homology (2 out of 680 bp).[5] Hybridization experiments performed with a probe from one of these chromosomes reveal the alpha-satellite fragments from both.[6]

A membrane-bound PCR[7] approach allowing the chromosomal origin (13 or 21) of alpha-satellite fragments detected by Southern blot hybridization is presented here. The experimental parameters that influence the PCR specificity also have implications for other purposes such as the detection of point mutations and the discrimination between the alleles of these models.

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II. MATERIALS AND METHODS

A. PRIMERS

Based on the nucleotide sequence of alpha-satellite subfamilies from chromosomes 13 and 21,[5] two pairs of primers (13A + 13B and 21A + 21B) were designed:

13A: 5′ TGATGTGTGTACCCAGCT 3′
13B: 5′ GCTATCCAAATATCCACT 3′
21A: 5′ TGATGTGTGTACCCAGCC 3′
21B: 5′ GCTATCCAAATATCCACC 3′

Each primer carries a chromosome-specific nucleotide at its 3′ end. They were resuspended in sterile distilled water to obtain stock solutions at 20 μ*M*.

B. BUFFERS AND REAGENTS

Distilled water was filtered through 0.22-μm filters and autoclaved at 120°C for 20 min before use. Small aliquots (1 ml) were reserved for PCR assays. Bovine Serum Albumine (BSA) was purchased from Boehringer Mannheim, diluted with water to 0.5 mg · ml^{-1}, and stored as 0.5-ml aliquots at –20°C. *Taq* DNA polymerase buffer 10 × concentrated was supplied by Promega: 500 m*M* KCl, 100 m*M* Tris-HCl, pH 8.8 at 25°C, 15 m*M* $MgCl_2$, 1% Triton X-100. Nucleotides, from Boehringer Mannheim, were separately resuspended in water to obtain stock solutions at 50 m*M*. Working solution (10 × concentrated) consists of a mix of the four dNTPs at 0.025 m*M* each. Diluted dNTPs are quite unstable and should be kept as small aliquots (20 μl) at –20°C. *Taq* DNA polymerase was from Promega, and Perfect Match polymerase enhancer was from Stratagene.

C. DNA TEMPLATES

The following somatic hybrids containing the specified human chromosome(s) in parentheses in a rodent genomic background were used: WA17 (human chromosome 21) was obtained from Dr. Devine;[8] HY124VT4, HY73DMT3 (both with human chromosome 21 in a hamster genomic background), RJ387.58T1, HY25T1 (both containing human chromosome 13), RJ387.91CT8 (chromosome 2), and HY129T14 (chromosome 14) were kindly provided by Dr. M. Rocchi; BCHE (human chromosomes 3, 4, 6, 8, 9, 10, 13, 14, 15, 16, 18, 19, Xp^+) and C35B2 (chromosome 11) were from Dr. N'Guyen Van Cong.

Cells were collected and washed twice with phosphate saline buffer (PBS) and lysed with 200 μg·ml^{-1} proteinase K (Appligène) in 0.5 *M* EDTA; 1% L-lauryl sarcosyl, pH 8.5 for 48 h at 50°C. The DNA was then purified by phenol extraction and ethanol precipitation according to standard methods.[9]

DNA was denatured in 0.5 *M* NaOH, 1.5 *M* NaCl for 5 min at room temperature and dotted onto nylon membrane (hybond N, Amersham). When dried, DNA was cross-linked to the membrane with UV light for 5 min. Small pieces (~1 × 1 mm) carrying ~100 ng of DNA were cut to obtain membrane-bound DNA templates for PCR.

Human genomic DNA was prepared embedded in low-melting agarose from blood or cultured cells as previously described.[6] Restriction enzyme hydrolysis, separation of DNA fragments by pulsed field gel electrophoresis (PFGE), Southern blotting and hybridization with the alpha-satellite probe α-RI 680, 368[5] from human chromosome 21 were performed as described[6] except that hybond N (Amersham) nylon membrane was used. After blotting, the filter was dried in an oven at 80°C and the DNA was cross-linked to the membrane with UV light (5 min on a UV table).

Pieces of membrane carrying the alpha-satellite fragments detected by hybridization were used as templates in PCR experiments.

D. PCR CONDITIONS

PCR experiments were performed in a PREM thermocycler as follows:

1. Prepare the sample mix (for 1 sample):

H_2O	9 µl
Taq DNA polymerase buffer 10 × concentrated	2 µl
Primer A, 20 µ*M*	1 µl
Primer B, 20 µ*M*	1 µl
BSA, 0.5 mg·ml^{-1}	2 µl

   Multiply these quantities by the number of PCR samples.

2. Distribute 15 µl of the sample mix in 0.5-ml reaction tubes, and add the DNA template (100 ng) and one drop of mineral oil to prevent evaporation.

3. Prepare the "enzyme mix" (for one sample):

H_2O	2.3 µl
Taq DNA polymerase (Promega), 5 U·µl^{-1}	0.5 µl
dNTPs 10 × concentrated	2.0 µl
Perfect Match polymerase enhancer, 1 U·µl^{-1}	0.2 µl

   Multiply by the number of samples and keep on ice.

4. Program the thermocycler as follows:

   1. 95°C, 5 min
   2. 59°C, 1 s
   3. Suspend the program
   4. 92°C, 5 s
   5. 59°C, 30 s
   6. Repeat from step 4, 40 times
   7. 72°C, 5 min

5. Place the tubes in the thermocycler and run the program. When the program is suspended at the annealing temperature after the initial denaturation, add 5 µl of "enzyme mix" to each reaction tube and continue the program.

For membrane-bound PCR, each piece of nylon filter carrying the DNA template was washed in 10 ml of H_2O for 30 min at 65°C and saturated in 200 µl of 1 × *Taq* DNA polymerase buffer containing 0.5 mg·ml^{-1} of BSA for 15 min at 65°C. The PCR conditions were the same except that 60 cycles were performed.

E. ANALYSIS OF PCR PRODUCTS

PCR products were analyzed in a 10% polyacrylamide gel prepared in TBE 0.5 × from a stock solution 38% acrylamide to 2% bisacrylamide. After 3 h of electrophoresis at 150 V, the DNA was stained by soaking the gel in an ethidium bromide solution (0.5 µg·ml^{-1}) for 30 min and visualized with UV light.

III. RESULTS

The PCR conditions allowing discrimination between the alpha-satellite subfamilies from chromosomes 13 and 21 were developed using the DNA from somatic hybrids containing one of these two chromosomes. A DNA fragment of the expected size (98 bp) was amplified with the two pairs of primers 13A + 13B and 21A + 21B. The nature of this DNA fragment was checked by Southern blot hybridization with the alpha-satellite probe used in this study (not shown).

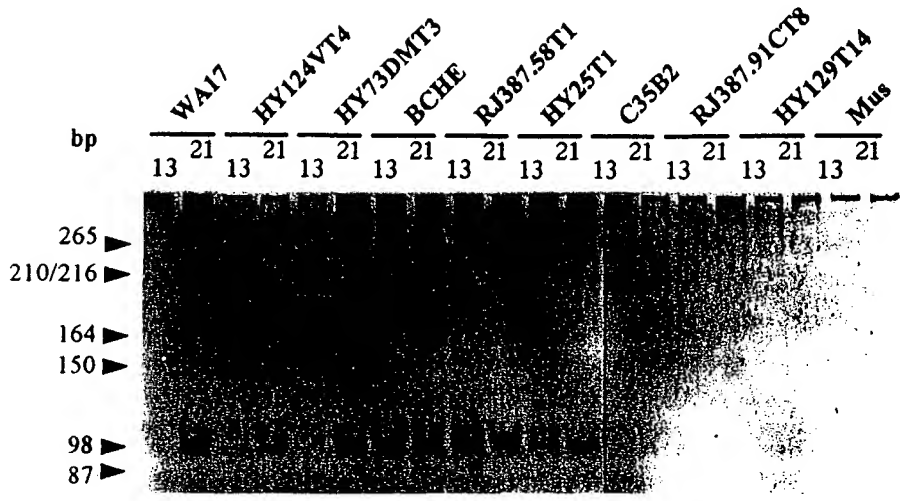

Figure 1. Membrane bound PCR with dot blots carrying the DNA from somatic hybrids noted at the top of the lanes. Numbers 13 and 21 indicate which pair of primers was used. 10 µl of the PCR products were loaded in a 10% polyacrylamide gel. The DNA was revealed by ethidium bromide staining and UV detection. (From Charlieu, J.-P., Murgue, B., Laurent, A.-M., Marçais, B., Bellis, M., and Roizès, G., *Genomics*, 14, 515, 1992. With permission.)

Membrane-bound PCR conditions were tested with dot blots carrying the DNA from the same somatic hybrids (Figure 1).

As for the experiment described above, the 98-bp amplified fragment was obtained with the chromosome 13-specific pair of primers (13A + 13B) only when using somatic hybrids containing chromosome 13, whereas the chromosome 21-specific pair of primers (21A + 21B) allowed the amplification of this DNA fragment in both chromosome 21- and 13-containing hybrids. The negative controls showed that this PCR product does not originate from the rodent genomic background or human chromosomes other than 13 and 21.

In order to test the possibility of determining the chromosomal origin of alpha-satellite fragments revealed by Southern blot hybridization, a CEPH (Centre d'Étude du Polymorphisme Humain, Paris) was used. Genomic DNA prepared in agarose plugs was digested with Bam HI, and the fragments were separated by PFGE. After hybridization and autoradiography, one band characterizing each allele determined by segregation analysis (A1, A2, B1, and B2 from the mother and C1, C2, D1, and D2 from the father)[6] was picked up and used in membrane-bound PCR (Figure 2). Alleles A1, A2, D1, and D2 were found to originate from chromosome 13, and alleles B1, B2, C1, and C2 from chromosome 21.

When analyzed by PFGE, the alpha-satellite Bam HI fragments were found to be quite variable in size. In addition, these alleles were found to segregate in a Mendelian fashion.[6] Alpha-satellite can therefore define a very informative centromeric marker when it is possible to distinguish by PCR the fragments from chromosomes 13 and 21.

IV. DISCUSSION

Annealing temperature is generally the main parameter considered for PCR specificity. In fact, several other parameters should be taken into account to obtain a specific PCR amplification. These are presented in this discussion, based on the example of PCR amplification of alpha-satellite subfamilies from chromosomes 13 and 21. The given values should be considered only as indications for the study of other models for which very stringent PCR conditions are needed.

It has been shown[1,10] that PCR amplification can be performed using primers containing up to 50% of mismatching nucleotides, but there is an absolute requirement for the correct base

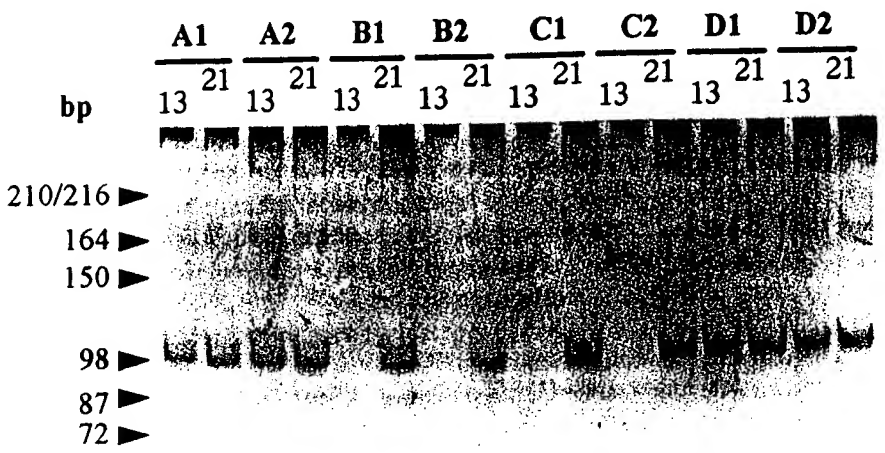

Figure 2. Membrane-bound PCR was performed with alpha-satellite primers on pieces of a Southern blot carrying the alpha-satellite Bam HI fragments revealed by hybridization. The PCR products, obtained with PCR conditions identical to those described in Figure 1, were analyzed in a 10% polyacrylamide gel. The CEPH family K1418 was used in this experiment. (From Charlieu, J.-P., Murgue, B., Laurent, A.-M., Marçais, B., Bellis, M., and Roizès, G., *Genomics*, 14, 515, 1992. With permission.)

pairing of their 3′ end. Thus, the main rule for the design of PCR primers allowing the discrimination between homologous DNA sequences is the *3′ position* of the mismatching nucleotide. In addition, Kwok et al.[1] have shown that all base mispairings do not work with the same efficiency. In particular, they have found that the T:G mismatch has no effect on PCR specificity. In the primary sequence of alpha-satellite DNA from chromosomes 13 and 21, the only differences are T-to-C changes, however.[5] The T:G mismatch (T on 13A and 13B primers and G on the chromosome 21 alpha-satellite DNA template) was found in this study to allow the correct discrimination between these two alpha-satellite DNA sequences: the 98-bp fragment was amplified with the pair of primers 13A + 13B only with the alpha-satellite template from chromosome 13. Thus, there might not be absolute rules in the nature of the mispairing nucleotide but this may depend on the model studied. Amplification of the 98-bp fragment from chromosome 13 with the chromosome 21-specific pair of primers could indicate either that the C:A mismatch (C on the 21A and 21B primers and A on the chromosome 13 alpha-satellite DNA sequence) does not affect PCR specificity or that the alpha-satellite subfamily of chromosome 21 is also present on chromosome 13.

The nucleotide concentration can also determine PCR specificity. In our laboratory, a "standard" PCR sample contains 0.2 m*M* of each dNTP. In these conditions, however, a 98-bp fragment was amplified in all cases with both chromosome 13 and 21 alpha-satellite-specific pairs of primers. Correct specificity was obtained by decreasing the nucleotide concentration to 2.5×10^{-3} m*M*.

$MgCl_2$ concentration also influences the PCR. We found that 1.5 m*M* was a good concentration for our system. Promega now provides *Taq* DNA polymerase buffer without $MgCl_2$, which can be added to give the desired concentration of this salt.

Another parameter affecting PCR specificity is the *Taq* DNA polymerase concentration. With the enzyme used, 2.5 U was the limit required to obtain reproducible and specific PCR amplification. We have also tested the thermostable DNA polymerase from Thermus flavis (Tfl polymerase), and we have found that the 98-bp alpha-satellite DNA fragment can be obtained with 1 or 0.5 U of enzyme, but results were not reproducible. If no specific amplification occurs in a given model, it is advisable to decrease the enzyme concentration or to test other enzymes.

The addition of chemicals such as tetramethylammonium chloride (TMAC)[11] or Perfect Match polymerase enhancer (Strategene) can also make it possible to obtain a specific

amplification. TMAC is generally used at 10^{-4} to 10^{-5} *M*. We have found that these conditions are too stringent, and we could not find the correct concentration for our system. The manufacturer recommands addition of 1 U of Perfect Match to the PCR sample. Again, we found the conditions too stringent for our case, and 0.2 U was sufficient. We recommend addition of Perfect Match with the enzyme and the dNTPs *after* the initial denaturation step in hot-start PCR. This product seems to be inactivated when submitted to a high temperature for a long time. We have also observed that non-specific amplification is reduced in these conditions. The mix of TMAC and Perfect Match can also be tried, but we found that they have a very strong effect on PCR when used together.

The stringency of the PCR is defined by a combination of these factors. We have described the conditions that work for our primers and in our hands. The study of other models or of our model in another laboratory and/or with another PCR machine will probably need some adjustments of the indicated values.

For membrane-bound PCR, we have tested several nylon supports. We have found that positively charged membranes are not convenient for our purpose. It seems that they have an extremely high capacity to capture the DNA and that, if amplification occurs, the PCR products are not released in the medium and cannot be detected. All uncharged nylon membranes we have tested present good qualities for membrane-bound PCR.

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REFERENCES

1. **Kwok, S., Kellog, D. E., McKinney, N., Spasic, D., Goda, L., and Sninsky, J. J.,** Effect of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies, *Nucleic Acids Res.*, 18, 999, 1991.
2. **Charlieu, J.-P., Murgue, B., Laurent, A.-M., Marçais, B., Bellis, M., and Roizès, G.,** Discrimination between alpha-satellite DNA sequences from chromosomes 21 and 13 by using polymerase chain reaction, *Genomics*, 14, 515, 1992.
3. **Manuelidis, L.,** Chromosomal localization of complex and simple repeated human DNAs, *Chromosoma*, 66, 1, 1978.
4. **Willard, H. F. and Waye, J. S.,** Hierarchical order in chromosome-specific human alpha-satellite DNA, *Trends Genet.*, 3, 192, 1987.
5. **Jørgensen, A. L., Bostock, C. J., and Bak, A. L.,** Homologous subfamilies of human alphoid repetitive DNA on different nucleolus organizing chromosomes, *Proc. Natl. Acad. Sci. U.S.A.*, 84, 1075, 1987.
6. **Marçais, B., Bellis, M., Gérard, A., Pagès, M., Boublik, Y., and Roizès, G.,** Structural organization and polymorphism of the alpha satellite DNA sequences of chromosomes 13 and 21 as revealed by pulsed field gel electrophoresis, *Hum. Genet.*, 86, 311, 1991.
7. **Kadokami, Y. and Lewis, R. V.,** Membrane-bound PCR, *Nucleic Acids Res.*, 18, 3082, 1990.
8. **Witek, M. P., Devine, E., Dutkowski, R., Blume, A., Mullikin-Kilpatrick, D., Miller, D. L., Brown, T., and Wrayne, W.,** Isolation of specific chromosomes and their DNA, *Gene Anal. Technol.*, 2, 16, 1985.
9. **Sambrook, J., Frisch, E. F., and Maniatis, T.,** *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
10. **Parker, J. D., Rabinovitch, P. S., and Burner, G. C.,** Targeted genewalking polymerase chain reaction, *Nucleic Acids Res.*, 9, 3055, 1991.
11. **Hung, T., Mak, K., and Fong, K.,** A specific enhancer for polymerase chain reaction, *Nucleic Acids Res.*, 18, 4953, 1990.

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